

PATENT APPLICATION

Of

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FOR

NOVEL ECDYSONE RECEPTOR-BASED INDUCIBLE GENE  
EXPRESSION SYSTEM

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**NOVEL ECDYSONE RECEPTOR-BASED INDUCIBLE GENE  
EXPRESSION SYSTEM**

This application is a Continuation-In-Part application and claims priority to co-  
5 pending International PCT application Serial number PCT/US01/09050, filed March 21,  
2001, which claims priority to both co-pending US provisional application Serial number  
60/191,355, filed March 22, 2000 and co-pending US provisional application Serial number  
60/269,799, filed February 20, 2001.

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**FIELD OF THE INVENTION**

This invention relates to the field of biotechnology or genetic engineering.  
Specifically, this invention relates to the field of gene expression. More specifically, this  
invention relates to a novel ecdysone receptor-based inducible gene expression system and  
15 methods of modulating the expression of a gene within a host cell using this inducible gene  
expression system.

**BACKGROUND OF THE INVENTION**

20 Various publications are cited herein, the disclosures of which are incorporated by  
reference in their entireties. However, the citation of any reference herein should not be  
construed as an admission that such reference is available as "Prior Art" to the instant  
application.

In the field of genetic engineering, precise control of gene expression is a valuable  
25 tool for studying, manipulating, and controlling development and other physiological  
processes. Gene expression is a complex biological process involving a number of specific  
protein-protein interactions. In order for gene expression to be triggered, such that it  
produces the RNA necessary as the first step in protein synthesis, a transcriptional activator  
must be brought into proximity of a promoter that controls gene transcription. Typically, the  
30 transcriptional activator itself is associated with a protein that has at least one DNA binding  
domain that binds to DNA binding sites present in the promoter regions of genes. Thus, for  
gene expression to occur, a protein comprising a DNA binding domain and a transactivation  
domain located at an appropriate distance from the DNA binding domain must be brought  
into the correct position in the promoter region of the gene.

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The traditional transgenic approach utilizes a cell-type specific promoter to drive the expression of a designed transgene. A DNA construct containing the transgene is first incorporated into a host genome. When triggered by a transcriptional activator, expression of the transgene occurs in a given cell type.

5 Another means to regulate expression of foreign genes in cells is through inducible promoters. Examples of the use of such inducible promoters include the PR1-a promoter, prokaryotic repressor-operator systems, immunosuppressive-immunophilin systems, and higher eukaryotic transcription activation systems such as steroid hormone receptor systems and are described below.

10 The PR1-a promoter from tobacco is induced during the systemic acquired resistance response following pathogen attack. The use of PR1-a may be limited because it often responds to endogenous materials and external factors such as pathogens, UV-B radiation, and pollutants. Gene regulation systems based on promoters induced by heat shock, interferon and heavy metals have been described (Wurn et al., 1986, Proc. Natl. Acad. Sci.  
15 USA 83:5414-5418; Arnheiter et al., 1990 Cell 62:51-61; Filmus et al., 1992 Nucleic Acids Research 20:27550-27560). However, these systems have limitations due to their effect on expression of non-target genes. These systems are also leaky.

Prokaryotic repressor-operator systems utilize bacterial repressor proteins and the unique operator DNA sequences to which they bind. Both the tetracycline ("Tet") and  
20 lactose ("Lac") repressor-operator systems from the bacterium *Escherichia coli* have been used in plants and animals to control gene expression. In the Tet system, tetracycline binds to the TetR repressor protein, resulting in a conformational change which releases the repressor protein from the operator which as a result allows transcription to occur. In the Lac system, a lac operon is activated in response to the presence of lactose, or synthetic analogs  
25 such as isopropyl-b-D-thiogalactoside. Unfortunately, the use of such systems is restricted by unstable chemistry of the ligands, *i.e.* tetracycline and lactose, their toxicity, their natural presence, or the relatively high levels required for induction or repression. For similar reasons, utility of such systems in animals is limited.

Immunosuppressive molecules such as FK506, rapamycin and cyclosporine A can  
30 bind to immunophilins FKBP12, cyclophilin, *etc.* Using this information, a general strategy has been devised to bring together any two proteins simply by placing FK506 on each of the two proteins or by placing FK506 on one and cyclosporine A on another one. A synthetic homodimer of FK506 (FK1012) or a compound resulted from fusion of FK506-cyclosporine (FKCsA) can then be used to induce dimerization of these molecules (Spencer et al., 1993,

*Science* 262:1019-24; Belshaw et al., 1996 *Proc Natl Acad Sci U S A* 93:4604-7). Gal4 DNA binding domain fused to FKBP12 and VP16 activator domain fused to cyclophilin, and FKCsA compound were used to show heterodimerization and activation of a reporter gene under the control of a promoter containing Gal4 binding sites. Unfortunately, this system includes immunosuppressants that can have unwanted side effects and therefore, limits its use for various mammalian gene switch applications.

Higher eukaryotic transcription activation systems such as steroid hormone receptor systems have also been employed. Steroid hormone receptors are members of the nuclear receptor superfamily and are found in vertebrate and invertebrate cells. Unfortunately, use of steroidal compounds that activate the receptors for the regulation of gene expression, particularly in plants and mammals, is limited due to their involvement in many other natural biological pathways in such organisms. In order to overcome such difficulties, an alternative system has been developed using insect ecdysone receptors (EcR).

Growth, molting, and development in insects are regulated by the ecdysone steroid hormone (molting hormone) and the juvenile hormones (Dhadialla, et al., 1998. *Annu. Rev. Entomol.* 43: 545-569). The molecular target for ecdysone in insects consists of at least ecdysone receptor (EcR) and ultraspiracle protein (USP). EcR is a member of the nuclear steroid receptor super family that is characterized by signature DNA and ligand binding domains, and an activation domain (Koelle et al. 1991, *Cell*, 67:59-77). EcR receptors are responsive to a number of steroidal compounds such as ponasterone A and muristerone A. Recently, non-steroidal compounds with ecdysteroid agonist activity have been described, including the commercially available insecticides tebufenozide and methoxyfenozide that are marketed world wide by Rohm and Haas Company (see International Patent Application No. PCT/EP96/00686 and US Patent 5,530,028). Both analogs have exceptional safety profiles to other organisms.

The insect ecdysone receptor (EcR) heterodimerizes with Ultraspiracle (USP), the insect homologue of the mammalian RXR, and binds ecdysteroids and ecdysone receptor response elements and activate transcription of ecdysone responsive genes (Riddiford et al., 2000, *Vitam Horm.*, 60:1-73). The EcR/USP/ligand complexes play important roles during insect development and reproduction. The EcR is a member of the steroid hormone receptor superfamily and has five modular domains, A/B (transactivation), C (DNA binding, heterodimerization), D (Hinge, heterodimerization), E (ligand binding, heterodimerization and transactivation) and F (transactivation) domains. Some of these domains such as A/B, C and E retain their function when they are fused to other proteins.

Tightly regulated inducible gene expression systems or "gene switches" are useful for various applications such as gene therapy, large scale production of proteins in cells, cell based high throughput screening assays, functional genomics and regulation of traits in transgenic plants and animals.

- 5 The first version of EcR-based gene switch used *Drosophila melanogaster* EcR (DmEcR) and *Mus musculus* RXR (MmRXR) and showed that these receptors in the presence of steroid, ponasteroneA, transactivate reporter genes in mammalian cell lines and transgenic mice (Christopherson et al., 1992, PNAS, 89:6314-6318; No et al., 1996, PNAS, 93:3346-3351). Later, Suhr et al. (1998, PNAS, 95:7999-8004) showed that non-steroidal  
10 ecdysone agonist, tebufenozide, induced high level of transactivation of reporter genes in mammalian cells through *Bombyx mori* EcR (BmEcR) in the absence of exogenous heterodimer partner.

- International Patent Applications No. PCT/US97/05330 (WO 97/38117) and PCT/US99/08381 (WO99/58155) disclose methods for modulating the expression of an  
15 exogenous gene in which a DNA construct comprising the exogenous gene and an ecdysone response element is activated by a second DNA construct comprising an ecdysone receptor that, in the presence of a ligand therefor, and optionally in the presence of a receptor capable of acting as a silent partner, binds to the ecdysone response element to induce gene expression. The ecdysone receptor of choice was isolated from *Drosophila melanogaster*.  
20 Typically, such systems require the presence of the silent partner, preferably retinoid X receptor (RXR), in order to provide optimum activation. In mammalian cells, insect ecdysone receptor (EcR) heterodimerizes with retinoid X receptor (RXR) and regulates expression of target genes in a ligand dependent manner. International Patent Application No. PCT/US98/14215 (WO 99/02683) discloses that the ecdysone receptor isolated from the silk  
25 moth *Bombyx mori* is functional in mammalian systems without the need for an exogenous dimer partner.

- U.S. Patent No. 6,265,173 B1 discloses that various members of the steroid/thyroid superfamily of receptors can combine with *Drosophila melanogaster* ultraspiracle receptor (USP) or fragments thereof comprising at least the dimerization domain of USP for use in a  
30 gene expression system. U.S. Patent No. 5,880,333 discloses a *Drosophila melanogaster* EcR and ultraspiracle (USP) heterodimer system used in plants in which the transactivation domain and the DNA binding domain are positioned on two different hybrid proteins. Unfortunately, these USP-based systems are constitutive in animal cells and therefore, are not effective for regulating reporter gene expression, particularly, in animal cells (for

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comparison, see Example 1.2, below).

In each of these cases, the transactivation domain and the DNA binding domain (either as native EcR as in International Patent Application No. PCT/US98/14215 or as modified EcR as in International Patent Application No. PCT/US97/05330) were  
5 incorporated into a single molecule and the other heterodimeric partners, either USP or RXR, were used in their native state.

Drawbacks of the above described EcR-based gene regulation systems include a considerable background activity in the absence of ligands and the non-applicability of these systems for use in both plants and animals (see U.S. Patent No. 5,880,333). Therefore, a  
10 need exists in the art for improved EcR-based systems to precisely modulate the expression of exogenous genes in both plants and animals. Such improved systems would be useful for applications such as gene therapy, large scale production of proteins and antibodies, cell-based high throughput screening assays, functional genomics and regulation of traits in transgenic animals. Improved systems that are simple, compact, and dependent on ligands  
15 that are relatively inexpensive, readily available, and of low toxicity to the host would prove useful for regulating biological systems.

For certain applications such as gene therapy, it may be desirable to have an inducible gene expression system that responds well to synthetic non-steroid ligands and at the same is insensitive to the natural steroids. Thus, improved systems that are simple,  
20 compact, and dependent on ligands that are relatively inexpensive, readily available, and of low toxicity to the host would prove useful for regulating biological systems.

Herein, Applicants describe an improved ecdysone receptor-based inducible gene expression system in which the transactivation and DNA binding domains are separated from each other by placing them on two different proteins results in greatly reduced background  
25 activity in the absence of a ligand and significantly increased activity over background in the presence of a ligand. This two-hybrid system is a significantly improved inducible gene expression modulation system compared to the two systems disclosed in applications PCT/US97/05330 and PCT/US98/14215. The two-hybrid system exploits the ability of a pair of interacting proteins to bring the transcription activation domain into a more favorable  
30 position relative to the DNA binding domain such that when the DNA binding domain binds to the DNA binding site on the gene, the transactivation domain more effectively activates the promoter (see, for example, U.S. Patent No. 5,283,173). Since the DNA binding and transactivation domains reside on two different molecules, the background activity in the absence of ligand is greatly reduced.

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A two-hybrid system also provides improved sensitivity to non-steroidal ligands for example, diacylhydrazines, when compared to steroidal ligands for example, ponasterone A ("PonA") or muristerone A ("MurA"). In addition, since transactivation based on EcR gene switches is often cell-line dependent, it is easier to tailor switching systems to obtain maximum transactivation capability for each application. Furthermore, the two-hybrid system avoids some side effects due to overexpression of RXR that often occur when unmodified RXR is used as a switching partner.

Thus Applicants' invention provides an improved ecdysone receptor-based inducible gene expression system for use in both prokaryotic and eukaryotic host cells in which ligand sensitivity and magnitude of transactivation may be selected as desired, depending upon the application.

The EcR is a member of the nuclear receptor superfamily and classified into subfamily 1, group H (referred to herein as "Group H nuclear receptors"). The members of each group share 40-60% amino acid identity in the E (ligand binding) domain (Laudet et al., A Unified Nomenclature System for the Nuclear Receptor Subfamily, 1999; Cell 97:161-163). In addition to the ecdysone receptor, other members of this nuclear receptor subfamily 1, group H include: ubiquitous receptor (UR), Orphan receptor 1 (OR-1), steroid hormone nuclear receptor 1 (NER-1), RXR interacting protein-15 (RIP-15), liver x receptor  $\beta$  (LXR $\beta$ ), steroid hormone receptor like protein (RLD-1), liver x receptor (LXR), liver x receptor  $\alpha$  (LXR $\alpha$ ), farnesoid x receptor (FXR), receptor interacting protein 14 (RIP-14), and farnesol receptor (HRR-1).

Given the close relatedness of ecdysone receptor to other Group H nuclear receptors, Applicants' invention also provides a two-hybrid Group H nuclear receptor-based gene expression system comprising ligand binding domains of a Group H nuclear receptor and its respective dimerization partner. Thus, Applicants' invention also provides a two-hybrid Group H nuclear receptor-based gene expression system for use in both prokaryotic and eukaryotic host cells in which ligand sensitivity and magnitude of transactivation may be selected as desired, depending upon the application.

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### **SUMMARY OF THE INVENTION**

The present invention relates to a novel ecdysone receptor-based inducible gene expression system, novel receptor polynucleotides and polypeptides for use in the novel inducible gene expression system, and methods of modulating the expression of a gene

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within a host cell using this inducible gene expression system. The present invention also relates to a novel Group H nuclear receptor-based gene expression system, novel receptor polynucleotides and polypeptides for use in the novel inducible gene expression system, and methods of modulating the expression of a gene within a host cell using this inducible gene expression system. In a specific embodiment, Applicants' invention relates to an improved Group H nuclear receptor-based inducible gene expression modulation system comprising a polynucleotide encoding a receptor polypeptide comprising a truncation mutation.

Specifically, the present invention relates to a gene expression modulation system comprising: a) a first gene expression cassette that is capable of being expressed in a host cell comprising a polynucleotide that encodes a first polypeptide comprising: i) a DNA-binding domain that recognizes a response element associated with a gene whose expression is to be modulated; and ii) a ligand binding domain comprising a ligand binding domain from a nuclear receptor; and b) a second gene expression cassette that is capable of being expressed in the host cell comprising a polynucleotide sequence that encodes a second polypeptide comprising: i) a transactivation domain; and ii) a ligand binding domain comprising a ligand binding domain from a nuclear receptor other than an ultraspiracle receptor. In a specific embodiment, the gene expression modulation system comprises a DNA binding domain and a transactivation domain from a polypeptide other than a Group H nuclear receptor, an ecdysone receptor, a Group B nuclear receptor, a retinoid X receptor, or an ultraspiracle receptor. In another specific embodiment, the ligand binding domains from the first polypeptide and the second polypeptide are different and dimerize.

In a specific embodiment, the ligand binding domain of the first polypeptide comprises a Group H nuclear receptor ligand binding domain. Preferably, the ligand binding domain of the first polypeptide comprises an ecdysone receptor (EcR) ligand binding domain.

In another specific embodiment, ligand binding domain of the second polypeptide comprises a Group B nuclear receptor ligand binding domain. Preferably, the ligand binding domain of the second polypeptide comprises a retinoid X receptor (RXR) ligand binding domain.

In a preferred embodiment, the ligand binding domain of the first polypeptide comprises a Group H nuclear receptor ligand binding domain and the ligand binding domain of the second polypeptide comprises a Group B nuclear receptor ligand binding domain. In another preferred embodiment, the ligand binding domain of the first polypeptide comprises an ecdysone receptor ligand binding domain and the ligand binding domain of the second polypeptide comprises a retinoid X receptor ligand binding domain.

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The present invention also relates to a gene expression modulation system according to the invention further comprising c) a third gene expression cassette comprising: i) a response element to which the DNA-binding domain of the first polypeptide binds; ii) a promoter that is activated by the transactivation domain of the second polypeptide; and iii) the gene whose expression is to be modulated.

The present invention also relates to an isolated polynucleotide encoding a truncated Group H nuclear receptor, truncated EcR, truncated Group B nuclear receptor, or a truncated RXR polypeptide, wherein the truncation mutation affects ligand binding activity or ligand sensitivity.

In particular, the present invention relates to an isolated polynucleotide encoding a truncated EcR or a truncated RXR polypeptide comprising a truncation mutation that reduces ligand binding activity or ligand sensitivity of said EcR or RXR polypeptide. In a specific embodiment, the present invention relates to an isolated polynucleotide encoding a truncated EcR or a truncated RXR polypeptide comprising a truncation mutation that reduces steroid binding activity or steroid sensitivity of said EcR or RXR polypeptide. In another specific embodiment, the present invention relates to an isolated polynucleotide encoding a truncated EcR or a truncated RXR polypeptide comprising a truncation mutation that reduces non-steroid binding activity or non-steroid sensitivity of said EcR or RXR polypeptide.

The present invention also relates to an isolated polynucleotide encoding a truncated EcR or a truncated RXR polypeptide comprising a truncation mutation that enhances ligand binding activity or ligand sensitivity of said EcR or RXR polypeptide. In a specific embodiment, the present invention relates to an isolated polynucleotide encoding a truncated EcR or a truncated RXR polypeptide comprising a truncation mutation that enhances steroid binding activity or steroid sensitivity of said EcR or RXR polypeptide. In another specific embodiment, the present invention relates to an isolated polynucleotide encoding a truncated EcR or a truncated RXR polypeptide comprising a truncation mutation that enhances non-steroid binding activity or non-steroid sensitivity of said EcR or RXR polypeptide.

The present invention also relates to an isolated polynucleotide encoding a truncated RXR polypeptide comprising a truncation mutation that increases ligand sensitivity of a heterodimer comprising the truncated retinoid X receptor polypeptide and a dimerization partner. In a specific embodiment, the dimerization partner is an ecdysone receptor polypeptide.

The present invention also relates to an isolated polypeptide encoded by a

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polynucleotide according to Applicants' invention. In particular, the present invention relates to an isolated truncated EcR or truncated RXR polypeptide comprising a truncation mutation, wherein the EcR or RXR polypeptide is encoded by a polynucleotide according to the invention.

5           Thus, the present invention also relates to an isolated truncated Group H nuclear receptor, truncated EcR, truncated Group B nuclear receptor, or truncated RXR polypeptide comprising a truncation mutation that affects ligand binding activity or ligand sensitivity of the Group H nuclear receptor polypeptide, the EcR polypeptide, the Group B nuclear receptor polypeptide, or the RXR polypeptide.

10           Applicants' invention also relates to methods of modulating gene expression in a host cell using a gene expression modulation system according to the invention. Specifically, Applicants' invention provides a method of modulating the expression of a gene in a host cell comprising the gene to be modulated comprising the steps of: a) introducing into the host cell a gene expression modulation system according to the invention; and b) introducing into  
15 the host cell a ligand that independently combines with the ligand binding domains of the first polypeptide and the second polypeptide of the gene expression modulation system; wherein the gene to be expressed is a component of a chimeric gene comprising: i) a response element comprising a domain to which the DNA binding domain from the first polypeptide binds; ii) a promoter that is activated by the transactivation domain of the  
20 second polypeptide; and iii) the gene whose expression is to be modulated, whereby a complex is formed comprising the ligand, the first polypeptide, and the second polypeptide, and whereby the complex modulates expression of the gene in the host cell.

          Applicants' invention also provides an isolated host cell comprising an inducible gene expression system according to the invention. The present invention also relates to an  
25 isolated host cell comprising a polynucleotide or polypeptide according to the invention. Accordingly, Applicants' invention also relates to a non-human organism comprising a host cell according to the invention.

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### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1: An ecdysone receptor-based gene expression system comprising a first gene expression cassette encoding a Gal4DBD-CfEcRDEF chimeric polypeptide and a second gene expression cassette encoding a VP16AD-MmRXRDEF-chimeric polypeptide; prepared

5 as described in Example 1 (switch 1.1).

Figure 2: An ecdysone receptor-based gene expression system comprising a first gene expression cassette encoding a Gal4DBD-CfEcRDEF chimeric polypeptide and a second gene expression cassette encoding a VP16AD-CfUSPDEF chimeric polypeptide; prepared as described in Example 1 (switch 1.2).

10 Figure 3: An ecdysone receptor-based gene expression system comprising a first gene expression cassette encoding a Gal4DBD-MmRXRDEF chimeric polypeptide and a second gene expression cassette encoding a VP16AD-CfEcRCDEF chimeric polypeptide; prepared as described in Example 1 (switch 1.3).

Figure 4: An ecdysone receptor-based gene expression system comprising a first gene  
15 expression cassette encoding a Gal4DBD-MmRXRDEF chimeric polypeptide and a second gene expression cassette encoding a VP16AD-DmEcRCDEF chimeric polypeptide; prepared as described in Example 1 (switch 1.4).

Figure 5: An ecdysone receptor-based gene expression system comprising a first gene expression cassette encoding a Gal4DBD-CfUSPDEF chimeric polypeptide and a second  
20 gene expression cassette encoding a VP16AD-CfEcRCDEF chimeric polypeptide; prepared as described in Example 1 (switch 1.5).

Figure 6: An ecdysone receptor-based gene expression system comprising a first gene expression cassette encoding a Gal4DBD-CfEcRDEF-VP16AD chimeric polypeptide; prepared as described in Example 1 (switch 1.6).

25 Figure 7: An ecdysone receptor-based gene expression system comprising a first gene expression cassette encoding a VP16AD-CfEcRCDEF chimeric polypeptide; prepared as described in Example 1 (switch 1.7).

Figure 8: An ecdysone receptor-based gene expression system comprising a first gene expression cassette encoding a VP16AD-DmEcRCDEF chimeric polypeptide and a second  
30 gene expression cassette encoding a MmRXR polypeptide; prepared as described in Example 1 (switch 1.8).

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Figure 9: An ecdysone receptor-based gene expression system comprising a first gene expression cassette encoding a VP16AD-CfEcRCDEF chimeric polypeptide and a second gene expression cassette encoding a MmRXR polypeptide; prepared as described in Example 1 (switch 1.9).

- 5 Figure 10: An ecdysone receptor-based gene expression system comprising a gene expression cassette encoding a Gal4DBD-CfEcRCDEF chimeric polypeptide; prepared as described in Example 1 (switch 1.10).

Figure 11: Expression data of GAL4CfEcRA/BCDEF, GAL4CfEcRCDEF, GAL4CfEcR1/2CDEF, GAL4CfEcRDEF, GAL4CfEcREF, GAL4CfEcRDE truncation  
10 mutants transfected into NIH3T3 cells along with VP16MmRXRDE, pFRLuc and pTKRL plasmid DNAs.

Figure 12: Expression data of GAL4CfEcRA/BCDEF, GAL4CfEcRCDEF, GAL4CfEcR1/2CDEF, GAL4CfEcRDEF, GAL4CfEcREF, GAL4CfEcRDE truncation  
15 mutants transfected into 3T3 cells along with VP16MmRXRE, pFRLuc and pTKRL plasmid DNAs.

Figure 13: Expression data of VP16MmRXRA/BCDEF, VP16MmRXRCDEF, VP16MmRXRDEF, VP16MmRXREF, VP16MmRXRBam-EF, VP16MmRXRAF2del constructs transfected into NIH3T3 cells along with GAL4CfEcRCDEF, pFRLuc and pTKRL plasmid DNAs.

20 Figure 14: Expression data of VP16MmRXRA/BCDEF, VP16MmRXRCDEF, VP16MmRXRDEF, VP16MmRXREF, VP16MmRXRBam-EF, VP16MmRXRAF2del constructs transfected into NIH3T3 cells along with GAL4CfEcRDEF, pFRLuc and pTKRL plasmid DNAs.

Figure 15: Expression data of various truncated CfEcR and MmRXR receptor pairs  
25 transfected into NIH3T3 cells along with GAL4CfEcRDEF, pFRLuc and pTKRL plasmid DNAs.

Figure 16: Expression data of CfEcR-DEF or CfEcR-CDEF in pGLIDA in combination with full length MmRXR, MmRXR-DEF or CfUSP-DEF in pB42AD transfected into yeast cells along with pSH118 reporter plasmid.

30 Figure 17: Fold induction expression data of CfEcR-DEF or CfEcR-CDEF in pGLIDA in combination with full length MmRXR, MmRXR-DEF or CfUSP-DEF in pB42AD transfected into yeast cells along with pSH118 reporter plasmid.

Figure 18: Expression data of CfEcR-DEF in pGLIDA in combination with MmRXR-DEF in pB42AD transfected into yeast cells along with pSH118 reporter plasmid in the presence

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of three steroidal ligands and four non-steroidal ligands.

Figure 19: Fold induction expression data of CfEcR-DEF in pGLIDA in combination with MmRXR-DEF in pB42AD transfected into yeast cells along with pSH118 reporter plasmid in the presence of three steroidal ligands and four non-steroidal ligands.

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### **DETAILED DESCRIPTION OF THE INVENTION**

Applicants describe herein an improved ecdysone receptor-based inducible gene expression system in which the transactivation and DNA binding domains are separated from each other by placing them on two different proteins results in greatly reduced background activity in the absence of a ligand and significantly increased activity over background in the presence of a ligand. This two-hybrid system is a significantly improved inducible gene expression modulation system compared to the two systems disclosed in applications PCT/US97/05330 and PCT/US98/14215. The two-hybrid system exploits the ability of a pair of interacting proteins to bring the transcription activation domain into a more favorable position relative to the DNA binding domain such that when the DNA binding domain binds to the DNA binding site on the gene, the transactivation domain more effectively activates the promoter (see, for example, U.S. Patent No. 5,283,173). Briefly, the two-hybrid gene expression system comprises two gene expression cassettes; the first encoding a DNA binding domain fused to a nuclear receptor polypeptide, and the second encoding a transactivation domain fused to a different nuclear receptor polypeptide. In the presence of ligand, the interaction of the first polypeptide with the second polypeptide effectively tethers the DNA binding domain to the transactivation domain. Since the DNA binding and transactivation domains reside on two different molecules, the background activity in the absence of ligand is greatly reduced.

Applicants' two-hybrid system also provides improved sensitivity to non-steroidal ligands for example, diacylhydrazines, when compared to steroidal ligands for example, ponasterone A ("PonA") or muristerone A ("MurA"). That is, when compared to steroids, the non-steroidal ligands provide higher activity at a lower concentration. In addition, since transactivation based on EcR gene switches is often cell-line dependent, it is easier to tailor switching systems to obtain maximum transactivation capability for each application. Furthermore, the two-hybrid system avoids some side effects due to overexpression of RXR that often occur when unmodified RXR is used as a switching partner. In a preferred two-hybrid system, native DNA binding and transactivation domains of EcR or RXR are

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eliminated and as a result, these hybrid molecules have less chance of interacting with other steroid hormone receptors present in the cell resulting in reduced side effects.

In a preferred embodiment, Applicants' improved ecdysone receptor-based inducible gene expression system comprises a truncation mutant of an ecdysone receptor or a retinoid X receptor (RXR) polypeptide that affects ligand binding activity or ligand sensitivity. This mutational effect may increase or reduce ligand binding activity or ligand sensitivity and may be steroid or non-steroid specific. Thus, Applicants' invention provides an improved ecdysone receptor-based inducible gene expression system useful for modulating expression of a gene of interest in a host cell. In a particularly desirable embodiment, Applicants' invention provides an inducible gene expression system that has a reduced level of background gene expression and responds to submicromolar concentrations of non-steroidal ligand. Thus, Applicants' novel inducible gene expression system and its use in methods of modulating gene expression in a host cell overcome the limitations of currently available inducible expression systems and provide the skilled artisan with an effective means to control gene expression.

The present invention provides a novel inducible gene expression system that can be used to modulate gene expression in both prokaryotic and eukaryotic host cells. Applicants' invention is useful for applications such as gene therapy, large scale production of proteins and antibodies, cell-based high throughput screening assays, orthogonal ligand screening assays, functional genomics, proteomics, metabolomics, and regulation of traits in transgenic organisms, where control of gene expression levels is desirable. An advantage of Applicants' invention is that it provides a means to regulate gene expression and to tailor expression levels to suit the user's requirements.

## 25 DEFINITIONS

In this disclosure, a number of terms and abbreviations are used. The following definitions are provided and should be helpful in understanding the scope and practice of the present invention.

In a specific embodiment, the term "about" or "approximately" means within 20%, preferably within 10%, more preferably within 5%, and even more preferably within 1% of a given value or range.

The term "substantially free" means that a composition comprising "A" (where "A" is a single protein, DNA molecule, vector, recombinant host cell, etc.) is substantially free of "B" (where "B" comprises one or more contaminating proteins, DNA molecules, vectors,

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etc.) when at least about 75% by weight of the proteins, DNA, vectors (depending on the category of species to which A and B belong) in the composition is "A". Preferably, "A" comprises at least about 90% by weight of the A+B species in the composition, most preferably at least about 99% by weight. It is also preferred that a composition, which is

5 substantially free of contamination, contain only a single molecular weight species having the activity or characteristic of the species of interest.

The term "isolated" for the purposes of the present invention designates a biological material (nucleic acid or protein) that has been removed from its original environment (the environment in which it is naturally present). For example, a polynucleotide present in the

10 natural state in a plant or an animal is not isolated, however the same polynucleotide separated from the adjacent nucleic acids in which it is naturally present, is considered "isolated". The term "purified" does not require the material to be present in a form exhibiting absolute purity, exclusive of the presence of other compounds. It is rather a relative definition.

15 For example, a polynucleotide present in the natural state in a plant or an animal is not isolated. The same polynucleotide separated from the adjacent nucleic acids in which it is naturally present. The term "purified" does not require the material to be present in a form exhibiting absolute purity, exclusive of the presence of other compounds. It is rather a relative definition.

20 A polynucleotide is in the "purified" state after purification of the starting material or of the natural material by at least one order of magnitude, preferably 2 or 3 and preferably 4 or 5 orders of magnitude.

A "nucleic acid" is a polymeric compound comprised of covalently linked subunits called nucleotides. Nucleic acid includes polyribonucleic acid (RNA) and

25 polydeoxyribonucleic acid (DNA), both of which may be single-stranded or double-stranded. DNA includes but is not limited to cDNA, genomic DNA, plasmids DNA, synthetic DNA, and semi-synthetic DNA. DNA may be linear, circular, or supercoiled.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or

30 deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of

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the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (e.g., restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal  
 5 convention of giving only the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

The term "fragment" will be understood to mean a nucleotide sequence of reduced  
 10 length relative to the reference nucleic acid and comprising, over the common portion, a nucleotide sequence identical to the reference nucleic acid. Such a nucleic acid fragment according to the invention may be, where appropriate, included in a larger polynucleotide of which it is a constituent. Such fragments comprise, or alternatively consist of, oligonucleotides ranging in length from at least 6, 8, 9, 10, 12, 15, 18, 20, 21, 22, 23, 24, 25,  
 15 30, 39, 40, 42, 45, 48, 50, 51, 54, 57, 60, 63, 66, 70, 75, 78, 80, 90, 100, 105, 120, 135, 150, 200, 300, 500, 720, 900, 1000 or 1500 consecutive nucleotides of a nucleic acid according to the invention.

As used herein, an "isolated nucleic acid fragment" is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered  
 20 nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

A "gene" refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acids. "Gene" also refers to a nucleic acid fragment that expresses a specific protein or polypeptide, including regulatory sequences  
 25 preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and/or coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived  
 30 from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. A chimeric gene may comprise coding sequences derived from different sources and/or regulatory sequences derived from different sources. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene or "heterologous" gene refers to a



gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

- 5 "Heterologous" DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell.

The term "genome" includes chromosomal as well as mitochondrial, chloroplast and viral DNA or RNA.

- 10 A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (see Sambrook *et al.*, 1989 *infra*). Hybridization and washing conditions are well known and exemplified in Sambrook, J., Fritsch, E. F. and Maniatis, T.
- 15 *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), particularly Chapter 11 and Table 11.1 therein (entirely incorporated herein by reference). The conditions of temperature and ionic strength determine the "stringency" of the hybridization.

- Stringency conditions can be adjusted to screen for moderately similar fragments,
- 20 such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a  $T_m$  of 55°, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization
- 25 conditions correspond to a higher  $T_m$ , *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest  $T_m$ , *e.g.*, 50% formamide, 5x or 6x SCC.

Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible.

- 30 The term "complementary" is used to describe the relationship between nucleotide bases that are capable of hybridizing to one another. For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine. Accordingly, the instant invention also includes isolated nucleic acid fragments that are complementary to the complete sequences as disclosed or used herein as well as those substantially similar nucleic

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acid sequences.

In a specific embodiment of the invention, polynucleotides are detected by employing hybridization conditions comprising a hybridization step at  $T_m$  of 55°C, and utilizing conditions as set forth above. In a preferred embodiment, the  $T_m$  is 60°C; in a more  
 5 preferred embodiment, the  $T_m$  is 63°C; in an even more preferred embodiment, the  $T_m$  is 65°C.

Post-hybridization washes also determine stringency conditions. One set of preferred conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 minutes (min), then repeated with 2X SSC, 0.5% SDS at 45°C for 30 minutes, and then  
 10 repeated twice with 0.2X SSC, 0.5% SDS at 50°C for 30 minutes. A more preferred set of stringent conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60°C. Another preferred set of highly stringent conditions uses two final washes in 0.1X SSC, 0.1% SDS at 65°C. Hybridization requires that the two nucleic acids comprise  
 15 complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible.

The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater  
 20 the value of  $T_m$  for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher  $T_m$ ) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating  $T_m$  have been derived (see Sambrook *et al.*, *supra*, 9.50-0.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches  
 25 becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook *et al.*, *supra*, 11.7-11.8).

In a specific embodiment of the invention, polynucleotides are detected by employing hybridization conditions comprising a hybridization step in less than 500 mM salt and at least 37 degrees Celsius, and a washing step in 2XSSPE at least 63 degrees Celsius. In  
 30 a preferred embodiment, the hybridization conditions comprise less than 200 mM salt and at least 37 degrees Celsius for the hybridization step. In a more preferred embodiment, the hybridization conditions comprise 2XSSPE and 63 degrees Celsius for both the hybridization and washing steps.

In one embodiment, the length for a hybridizable nucleic acid is at least about 10

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nucleotides. Preferable a minimum length for a hybridizable nucleic acid is at least about 15 nucleotides; more preferably at least about 20 nucleotides; and most preferably the length is at least 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as length of the probe.

The term "probe" refers to a single-stranded nucleic acid molecule that can base pair with a complementary single stranded target nucleic acid to form a double-stranded molecule.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of at least 18 nucleotides, that is hybridizable to a genomic DNA molecule, a cDNA molecule, a plasmid DNA or an mRNA molecule. Oligonucleotides can be labeled, *e.g.*, with  $^{32}\text{P}$ -nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. A labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. Oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of a nucleic acid, or to detect the presence of a nucleic acid. An oligonucleotide can also be used to form a triple helix with a DNA molecule. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer. Accordingly, oligonucleotides can be prepared with non-naturally occurring phosphoester analog bonds, such as thioester bonds, etc.

A "primer" is an oligonucleotide that hybridizes to a target nucleic acid sequence to create a double stranded nucleic acid region that can serve as an initiation point for DNA synthesis under suitable conditions. Such primers may be used in a polymerase chain reaction.

"Polymerase chain reaction" is abbreviated PCR and means an *in vitro* method for enzymatically amplifying specific nucleic acid sequences. PCR involves a repetitive series of temperature cycles with each cycle comprising three stages: denaturation of the template nucleic acid to separate the strands of the target molecule, annealing a single stranded PCR oligonucleotide primer to the template nucleic acid, and extension of the annealed primer(s) by DNA polymerase. PCR provides a means to detect the presence of the target molecule and, under quantitative or semi-quantitative conditions, to determine the relative amount of that target molecule within the starting pool of nucleic acids.

"Reverse transcription-polymerase chain reaction" is abbreviated RT-PCR and means an *in vitro* method for enzymatically producing a target cDNA molecule or molecules from an RNA molecule or molecules, followed by enzymatic amplification of a specific nucleic

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acid sequence or sequences within the target cDNA molecule or molecules as described above. RT-PCR also provides a means to detect the presence of the target molecule and, under quantitative or semi-quantitative conditions, to determine the relative amount of that target molecule within the starting pool of nucleic acids.

- 5 A DNA "coding sequence" is a double-stranded DNA sequence that is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. "Suitable regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or
- 10 stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing site, effector binding site and stem-loop structure. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited
- 15 to, prokaryotic sequences, cDNA from mRNA, genomic DNA sequences, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

- "Open reading frame" is abbreviated ORF and means a length of nucleic acid
- 20 sequence, either DNA, cDNA or RNA, that comprises a translation start signal or initiation codon, such as an ATG or AUG, and a termination codon and can be potentially translated into a polypeptide sequence.

- The term "head-to-head" is used herein to describe the orientation of two polynucleotide sequences in relation to each other. Two polynucleotides are positioned in a
- 25 head-to-head orientation when the 5' end of the coding strand of one polynucleotide is adjacent to the 5' end of the coding strand of the other polynucleotide, whereby the direction of transcription of each polynucleotide proceeds away from the 5' end of the other polynucleotide. The term "head-to-head" may be abbreviated (5')-to-(5') and may also be indicated by the symbols ( $\leftarrow \rightarrow$ ) or ( $3' \leftarrow 5' 5' \rightarrow 3'$ ).

- 30 The term "tail-to-tail" is used herein to describe the orientation of two polynucleotide sequences in relation to each other. Two polynucleotides are positioned in a tail-to-tail orientation when the 3' end of the coding strand of one polynucleotide is adjacent to the 3' end of the coding strand of the other polynucleotide, whereby the direction of transcription of each polynucleotide proceeds toward the other polynucleotide. The term "tail-to-tail" may be

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abbreviated (3')-to-(3') and may also be indicated by the symbols ( $\rightarrow \leftarrow$ ) or ( $5' \rightarrow 3' 3' \leftarrow 5'$ ).

The term "head-to-tail" is used herein to describe the orientation of two polynucleotide sequences in relation to each other. Two polynucleotides are positioned in a head-to-tail orientation when the 5' end of the coding strand of one polynucleotide is  
 5 adjacent to the 3' end of the coding strand of the other polynucleotide, whereby the direction of transcription of each polynucleotide proceeds in the same direction as that of the other polynucleotide. The term "head-to-tail" may be abbreviated (5')-to-(3') and may also be indicated by the symbols ( $\rightarrow \rightarrow$ ) or ( $5' \rightarrow 3' 5' \rightarrow 3'$ ).

The term "downstream" refers to a nucleotide sequence that is located 3' to reference  
 10 nucleotide sequence. In particular, downstream nucleotide sequences generally relate to sequences that follow the starting point of transcription. For example, the translation initiation codon of a gene is located downstream of the start site of transcription.

The term "upstream" refers to a nucleotide sequence that is located 5' to reference nucleotide sequence. In particular, upstream nucleotide sequences generally relate to  
 15 sequences that are located on the 5' side of a coding sequence or starting point of transcription. For example, most promoters are located upstream of the start site of transcription.

The terms "restriction endonuclease" and "restriction enzyme" refer to an enzyme that binds and cuts within a specific nucleotide sequence within double stranded DNA.  
 20 "Homologous recombination" refers to the insertion of a foreign DNA sequence into another DNA molecule, e.g., insertion of a vector in a chromosome. Preferably, the vector targets a specific chromosomal site for homologous recombination. For specific homologous recombination, the vector will contain sufficiently long regions of homology to sequences of the chromosome to allow complementary binding and incorporation of the vector into the  
 25 chromosome. Longer regions of homology, and greater degrees of sequence similarity, may increase the efficiency of homologous recombination.

Several methods known in the art may be used to propagate a polynucleotide according to the invention. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As  
 30 described herein, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors, to name but a few.

A "vector" is any means for the cloning of and/or transfer of a nucleic acid into a

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host cell. A vector may be a replicon to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (e.g., plasmid, phage, cosmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, *i.e.*, capable of replication under its own control. The term "vector" includes both viral and nonviral means for introducing the nucleic acid into a cell *in vitro*, *ex vivo* or *in vivo*. A large number of vectors known in the art may be used to manipulate nucleic acids, incorporate response elements and promoters into genes, etc. Possible vectors include, for example, plasmids or modified viruses including, for example bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives, or the Bluescript vector. For example, the insertion of the DNA fragments corresponding to response elements and promoters into a suitable vector can be accomplished by ligating the appropriate DNA fragments into a chosen vector that has complementary cohesive termini. Alternatively, the ends of the DNA molecules may be enzymatically modified or any site may be produced by ligating nucleotide sequences (linkers) into the DNA termini. Such vectors may be engineered to contain selectable marker genes that provide for the selection of cells that have incorporated the marker into the cellular genome. Such markers allow identification and/or selection of host cells that incorporate and express the proteins encoded by the marker.

Viral vectors, and particularly retroviral vectors, have been used in a wide variety of gene delivery applications in cells, as well as living animal subjects. Viral vectors that can be used include but are not limited to retrovirus, adeno-associated virus, pox, baculovirus, vaccinia, herpes simplex, Epstein-Barr, adenovirus, geminivirus, and caulimovirus vectors. Non-viral vectors include plasmids, liposomes, electrically charged lipids (cytofectins), DNA-protein complexes, and biopolymers. In addition to a nucleic acid, a vector may also comprise one or more regulatory regions, and/or selectable markers useful in selecting, measuring, and monitoring nucleic acid transfer results (transfer to which tissues, duration of expression, etc.).

The term "plasmid" refers to an extra chromosomal element often carrying a gene that is not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear, circular, or supercoiled, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene

product along with appropriate 3' untranslated sequence into a cell.

A "cloning vector" is a "replicon", which is a unit length of a nucleic acid, preferably DNA, that replicates sequentially and which comprises an origin of replication, such as a plasmid, phage or cosmid, to which another nucleic acid segment may be attached so as to  
 5 bring about the replication of the attached segment. Cloning vectors may be capable of replication in one cell type and expression in another ("shuttle vector").

Vectors may be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA  
 10 vector transporter (see, *e.g.*, Wu et al., 1992, J. Biol. Chem. 267:963-967; Wu and Wu, 1988, J. Biol. Chem. 263:14621-14624; and Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990).

A polynucleotide according to the invention can also be introduced *in vivo* by lipofection. For the past decade, there has been increasing use of liposomes for encapsulation and  
 15 transfection of nucleic acids *in vitro*. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner et al., 1987. PNAS 84:7413; Mackey, et al., 1988. Proc. Natl. Acad. Sci. U.S.A. 85:8027-8031; and Ulmer et al., 1993. Science 259:1745-1748). The use of cationic lipids may promote encapsulation of negatively charged  
 20 nucleic acids, and also promote fusion with negatively charged cell membranes (Felgner and Ringold, 1989. Science 337:387-388). Particularly useful lipid compounds and compositions for transfer of nucleic acids are described in International Patent Publications WO95/18863 and WO96/17823, and in U.S. Patent No. 5,459,127. The use of lipofection to introduce exogenous genes into the specific organs *in vivo* has certain practical advantages. Molecular targeting of  
 25 liposomes to specific cells represents one area of benefit. It is clear that directing transfection to particular cell types would be particularly preferred in a tissue with cellular heterogeneity, such as pancreas, liver, kidney, and the brain. Lipids may be chemically coupled to other molecules for the purpose of targeting (Mackey, et al., 1988, *supra*). Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to  
 30 liposomes chemically.

Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, such as a cationic oligopeptide (*e.g.*, WO95/21931), peptides derived from DNA binding proteins (*e.g.*, WO96/25508), or a cationic polymer (*e.g.*, WO95/21931).

It is also possible to introduce a vector *in vivo* as a naked DNA plasmid (see U.S. Patents 5,693,622, 5,589,466 and 5,580,859). Receptor-mediated DNA delivery approaches can also be used (Curiel et al., 1992. Hum. Gene Ther. 3:147-154; and Wu and Wu, 1987. J. Biol. Chem. 262:4429-4432).

- 5       The term "transfection" means the uptake of exogenous or heterologous RNA or DNA by a cell. A cell has been "transfected" by exogenous or heterologous RNA or DNA when such RNA or DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous RNA or DNA when the transfected RNA or DNA effects a phenotypic change. The transforming RNA or DNA can be integrated (covalently linked)  
10 into chromosomal DNA making up the genome of the cell.

"Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

- 15       The term "genetic region" will refer to a region of a nucleic acid molecule or a nucleotide sequence that comprises a gene encoding a polypeptide.

In addition, the recombinant vector comprising a polynucleotide according to the invention may include one or more origins for replication in the cellular hosts in which their amplification or their expression is sought, markers or selectable markers.

- 20       The term "selectable marker" means an identifying factor, usually an antibiotic or chemical resistance gene, that is able to be selected for based upon the marker gene's effect, *i.e.*, resistance to an antibiotic, resistance to a herbicide, colorimetric markers, enzymes, fluorescent markers, and the like, wherein the effect is used to track the inheritance of a nucleic acid of interest and/or to identify a cell or organism that has inherited the nucleic acid  
25 of interest. Examples of selectable marker genes known and used in the art include: genes providing resistance to ampicillin, streptomycin, gentamycin, kanamycin, hygromycin, bialaphos herbicide, sulfonamide, and the like; and genes that are used as phenotypic markers, *i.e.*, anthocyanin regulatory genes, isopentanyl transferase gene, and the like.

- 30       The term "reporter gene" means a nucleic acid encoding an identifying factor that is able to be identified based upon the reporter gene's effect, wherein the effect is used to track the inheritance of a nucleic acid of interest, to identify a cell or organism that has inherited the nucleic acid of interest, and/or to measure gene expression induction or transcription. Examples of reporter genes known and used in the art include: luciferase (Luc), green

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fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT),  $\beta$ -galactosidase (LacZ),  $\beta$ -glucuronidase (Gus), and the like. Selectable marker genes may also be considered reporter genes.

“Promoter” refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters that cause a gene to be expressed in most cell types at most times are commonly referred to as “constitutive promoters”. Promoters that cause a gene to be expressed in a specific cell type are commonly referred to as “cell-specific promoters” or “tissue-specific promoters”. Promoters that cause a gene to be expressed at a specific stage of development or cell differentiation are commonly referred to as “developmentally-specific promoters” or “cell differentiation-specific promoters”. Promoters that are induced and cause a gene to be expressed following exposure or treatment of the cell with an agent, biological molecule, chemical, ligand, light, or the like that induces the promoter are commonly referred to as “inducible promoters” or “regulatable promoters”. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

A “promoter sequence” is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is “under the control” of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced (if the coding sequence contains introns) and translated into the protein encoded by the coding sequence.

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“Transcriptional and translational control sequences” are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

- 5       The term “response element” means one or more cis-acting DNA elements which confer responsiveness on a promoter mediated through interaction with the DNA-binding domains of the first chimeric gene. This DNA element may be either palindromic (perfect or imperfect) in its sequence or composed of sequence motifs or half sites separated by a variable number of nucleotides. The half sites can be similar or identical and arranged as
- 10 either direct or inverted repeats or as a single half site or multimers of adjacent half sites in tandem. The response element may comprise a minimal promoter isolated from different organisms depending upon the nature of the cell or organism into which the response element will be incorporated. The DNA binding domain of the first hybrid protein binds, in the presence or absence of a ligand, to the DNA sequence of a response element to initiate or
- 15 suppress transcription of downstream gene(s) under the regulation of this response element. Examples of DNA sequences for response elements of the natural ecdysone receptor include: RRGG/TTCANTGAC/ACY (see Cherbas L., et. al., (1991), *Genes Dev.* 5, 120-131); AGGTCAN<sub>(n)</sub>AGGTCA, where N<sub>(n)</sub> can be one or more spacer nucleotides (see D'Avino PP., et. al., (1995), *Mol. Cell. Endocrinol.* 113, 1-9); and GGGTTGAATGAATTT
- 20 (see Antoniewski C., et. al., (1994). *Mol. Cell Biol.* 14, 4465-4474).

- The term “operably linked” refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional
- 25 control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

- The term “expression”, as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from a nucleic acid or polynucleotide. Expression may also refer to translation of mRNA into a protein or
- 30 polypeptide.

The terms “cassette”, “expression cassette” and “gene expression cassette” refer to a segment of DNA that can be inserted into a nucleic acid or polynucleotide at specific restriction sites or by homologous recombination. The segment of DNA comprises a polynucleotide that encodes a polypeptide of interest, and the cassette and restriction sites are

designed to ensure insertion of the cassette in the proper reading frame for transcription and translation. "Transformation cassette" refers to a specific vector comprising a polynucleotide that encodes a polypeptide of interest and having elements in addition to the polynucleotide that facilitate transformation of a particular host cell. Cassettes, expression cassettes, gene

5 expression cassettes and transformation cassettes of the invention may also comprise elements that allow for enhanced expression of a polynucleotide encoding a polypeptide of interest in a host cell. These elements may include, but are not limited to: a promoter, a minimal promoter, an enhancer, a response element, a terminator sequence, a polyadenylation sequence, and the like.

10 For purposes of this invention, the term "gene switch" refers to the combination of a response element associated with a promoter, and an EcR based system which, in the presence of one or more ligands, modulates the expression of a gene into which the response element and promoter are incorporated.

The terms "modulate" and "modulates" mean to induce, reduce or inhibit nucleic  
15 acid or gene expression, resulting in the respective induction, reduction or inhibition of protein or polypeptide production.

The plasmids or vectors according to the invention may further comprise at least one promoter suitable for driving expression of a gene in a host cell. The term "expression vector" means a vector, plasmid or vehicle designed to enable the expression of an inserted  
20 nucleic acid sequence following transformation into the host. The cloned gene, i.e., the inserted nucleic acid sequence, is usually placed under the control of control elements such as a promoter, a minimal promoter, an enhancer, or the like. Initiation control regions or promoters, which are useful to drive expression of a nucleic acid in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving  
25 these genes is suitable for the present invention including but not limited to: viral promoters, bacterial promoters, animal promoters, mammalian promoters, synthetic promoters, constitutive promoters, tissue specific promoter, developmental specific promoters, inducible promoters, light regulated promoters; *CYC1*, *HIS3*, *GAL1*, *GAL4*, *GAL10*, *ADHI*, *PGK*, *PHO5*, *GAPDH*, *ADC1*, *TRP1*, *URA3*, *LEU2*, *ENO*, *TPI*, alkaline phosphatase promoters  
30 (useful for expression in *Saccharomyces*); *AOX1* promoter (useful for expression in *Pichia*); b-lactamase, *lac*, *ara*, *tet*, *trp*, *lP<sub>L</sub>*, *lP<sub>R</sub>*, *T7*, *tac*, and *trc* promoters (useful for expression in *Escherichia coli*); light regulated-, seed specific-, pollen specific-, ovary specific-, pathogenesis or disease related-, cauliflower mosaic virus 35S, CMV 35S minimal, cassava vein mosaic virus (CsVMV), chlorophyll a/b binding protein, ribulose 1, 5-bisphosphate

carboxylase, shoot-specific, root specific, chitinase, stress inducible, rice tungro bacilliform virus, plant super-promoter, potato leucine aminopeptidase, nitrate reductase, mannopine synthase, nopaline synthase, ubiquitin, zein protein, and anthocyanin promoters (useful for expression in plant cells); animal and mammalian promoters known in the art include, but are

5 not limited to, the SV40 early (SV40e) promoter region, the promoter contained in the 3' long terminal repeat (LTR) of Rous sarcoma virus (RSV), the promoters of the E1A or major late promoter (MLP) genes of adenoviruses (Ad), the cytomegalovirus (CMV) early promoter, the herpes simplex virus (HSV) thymidine kinase (TK) promoter, a baculovirus IE1 promoter, an elongation factor 1 alpha (EF1) promoter, a phosphoglycerate kinase (PGK)

10 promoter, a ubiquitin (Ubc) promoter, an albumin promoter, the regulatory sequences of the mouse metallothionein-L promoter and transcriptional control regions, the ubiquitous promoters (HPRT, vimentin,  $\alpha$ -actin, tubulin and the like), the promoters of the intermediate filaments (desmin, neurofilaments, keratin, GFAP, and the like), the promoters of therapeutic genes (of the MDR, CFTR or factor VIII type, and the like), pathogenesis or disease related-

15 promoters, and promoters that exhibit tissue specificity and have been utilized in transgenic animals, such as the elastase I gene control region which is active in pancreatic acinar cells; insulin gene control region active in pancreatic beta cells, immunoglobulin gene control region active in lymphoid cells, mouse mammary tumor virus control region active in testicular, breast, lymphoid and mast cells; albumin gene, Apo AI and Apo AII control

20 regions active in liver, alpha-fetoprotein gene control region active in liver, alpha 1-antitrypsin gene control region active in the liver, beta-globin gene control region active in myeloid cells, myelin basic protein gene control region active in oligodendrocyte cells in the brain, myosin light chain-2 gene control region active in skeletal muscle, and gonadotropic releasing hormone gene control region active in the hypothalamus, pyruvate kinase promoter,

25 villin promoter, promoter of the fatty acid binding intestinal protein, promoter of the smooth muscle cell  $\alpha$ -actin, and the like. In a preferred embodiment of the invention, the promoter is selected from the group consisting of a cauliflower mosaic virus 35S promoter, a cassava vein mosaic virus promoter, and a cauliflower mosaic virus 35S minimal promoter, an elongation factor 1 alpha (EF1) promoter, a phosphoglycerate kinase (PGK) promoter, a

30 ubiquitin (Ubc) promoter, and an albumin promoter. In addition, these expression sequences may be modified by addition of enhancer or regulatory sequences and the like.

Enhancers that may be used in embodiments of the invention include but are not limited to: tobacco mosaic virus enhancer, cauliflower mosaic virus 35S enhancer, tobacco etch virus enhancer, ribulose 1, 5-bisphosphate carboxylase enhancer, rice tungro bacilliform

virus enhancer, an SV40 enhancer, a cytomegalovirus (CMV) enhancer, an elongation factor 1 (EF1) enhancer, yeast enhancers, other plant and viral gene enhancers, and the like.

Termination control regions, *i.e.*, terminator or polyadenylation sequences, may also be derived from various genes native to the preferred hosts. Optionally, a termination site  
 5 may be unnecessary, however, it is most preferred if included. In a preferred embodiment of the invention, the termination control region may be comprise or be derived from a synthetic sequence, synthetic polyadenylation signal, an SV40 late polyadenylation signal, an SV40 polyadenylation signal, a bovine growth hormone (BGH) polyadenylation signal, nopaline synthase (nos), cauliflower mosaic virus (CaMV), octopine synthase (ocs), Agrocateum,  
 10 viral, and plant terminator sequences, or the like.

The terms "3' non-coding sequences" or "3' untranslated region (UTR)" refer to DNA sequences located downstream (3') of a coding sequence and may comprise polyadenylation [poly(A)] recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation  
 15 signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

"Regulatory region" means a nucleic acid sequence which regulates the expression of a second nucleic acid sequence. A regulatory region may include sequences which are naturally responsible for expressing a particular nucleic acid (a homologous region) or may  
 20 include sequences of a different origin that are responsible for expressing different proteins or even synthetic proteins (a heterologous region). In particular, the sequences can be sequences of prokaryotic, eukaryotic, or viral genes or derived sequences that stimulate or repress transcription of a gene in a specific or non-specific manner and in an inducible or non-inducible manner. Regulatory regions include origins of replication, RNA splice sites,  
 25 promoters, enhancers, transcriptional termination sequences, and signal sequences which direct the polypeptide into the secretory pathways of the target cell.

A regulatory region from a "heterologous source" is a regulatory region that is not naturally associated with the expressed nucleic acid. Included among the heterologous regulatory regions are regulatory regions from a different species, regulatory regions from a  
 30 different gene, hybrid regulatory sequences, and regulatory sequences which do not occur in nature, but which are designed by one having ordinary skill in the art.

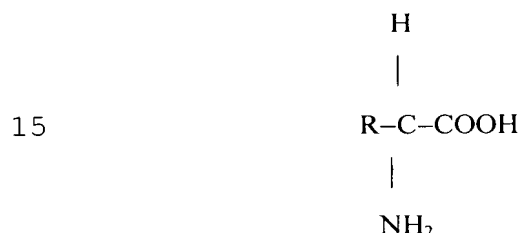
"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence

derived from post-transcriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a double-stranded DNA that is complementary to and derived from mRNA. "Sense" RNA refers to RNA transcript that

5 includes the mRNA and so can be translated into protein by the cell. "Antisense RNA" refers to a RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene. The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, or the coding sequence. "Functional RNA" refers to antisense RNA,

10 ribozyme RNA, or other RNA that is not translated yet has an effect on cellular processes.

A "polypeptide" is a polymeric compound comprised of covalently linked amino acid residues. Amino acids have the following general structure:



Amino acids are classified into seven groups on the basis of the side chain R: (1) aliphatic side chains, (2) side chains containing a hydroxylic (OH) group, (3) side chains containing

20 sulfur atoms, (4) side chains containing an acidic or amide group, (5) side chains containing a basic group, (6) side chains containing an aromatic ring, and (7) proline, an imino acid in which the side chain is fused to the amino group. A polypeptide of the invention preferably comprises at least about 14 amino acids.

A "protein" is a polypeptide that performs a structural or functional role in a living

25 cell.

An "isolated polypeptide" or "isolated protein" is a polypeptide or protein that is substantially free of those compounds that are normally associated therewith in its natural state (e.g., other proteins or polypeptides, nucleic acids, carbohydrates, lipids). "Isolated" is not meant to exclude artificial or synthetic mixtures with other compounds, or the presence of

30 impurities which do not interfere with biological activity, and which may be present, for example, due to incomplete purification, addition of stabilizers, or compounding into a pharmaceutically acceptable preparation.

"Fragment" of a polypeptide according to the invention will be understood to mean a polypeptide whose amino acid sequence is shorter than that of the reference polypeptide and

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which comprises, over the entire portion with these reference polypeptides, an identical amino acid sequence. Such fragments may, where appropriate, be included in a larger polypeptide of which they are a part. Such fragments of a polypeptide according to the invention may have a length of at least 2, 3, 4, 5, 6, 8, 10, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 25, 26, 30, 35, 40, 45, 50, 100, 200, 240, or 300 amino acids.

A "variant" of a polypeptide or protein is any analogue, fragment, derivative, or mutant which is derived from a polypeptide or protein and which retains at least one biological property of the polypeptide or protein. Different variants of the polypeptide or protein may exist in nature. These variants may be allelic variations characterized by differences in the nucleotide sequences of the structural gene coding for the protein, or may involve differential splicing or post-translational modification. The skilled artisan can produce variants having single or multiple amino acid substitutions, deletions, additions, or replacements. These variants may include, *inter alia*: (a) variants in which one or more amino acid residues are substituted with conservative or non-conservative amino acids, (b) variants in which one or more amino acids are added to the polypeptide or protein, (c) variants in which one or more of the amino acids includes a substituent group, and (d) variants in which the polypeptide or protein is fused with another polypeptide such as serum albumin. The techniques for obtaining these variants, including genetic ( suppressions, deletions, mutations, etc.), chemical, and enzymatic techniques, are known to persons having ordinary skill in the art. A variant polypeptide preferably comprises at least about 14 amino acids.

A "heterologous protein" refers to a protein not naturally produced in the cell.

A "mature protein" refers to a post-translationally processed polypeptide; i.e., one from which any pre- or propeptides present in the primary translation product have been removed. "Precursor" protein refers to the primary product of translation of mRNA; i.e., with pre- and propeptides still present. Pre- and propeptides may be but are not limited to intracellular localization signals.

The term "signal peptide" refers to an amino terminal polypeptide preceding the secreted mature protein. The signal peptide is cleaved from and is therefore not present in the mature protein. Signal peptides have the function of directing and translocating secreted proteins across cell membranes. Signal peptide is also referred to as signal protein.

A "signal sequence" is included at the beginning of the coding sequence of a protein to be expressed on the surface of a cell. This sequence encodes a signal peptide, N-terminal to the mature polypeptide, that directs the host cell to translocate the polypeptide. The term

"translocation signal sequence" is used herein to refer to this sort of signal sequence. Translocation signal sequences can be found associated with a variety of proteins native to eukaryotes and prokaryotes, and are often functional in both types of organisms.

The term "homology" refers to the percent of identity between two polynucleotide or  
 5 two polypeptide moieties. The correspondence between the sequence from one moiety to another can be determined by techniques known to the art. For example, homology can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs. Alternatively, homology can be determined by hybridization of polynucleotides  
 10 under conditions that form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s) and size determination of the digested fragments.

As used herein, the term "homologous" in all its grammatical forms and spelling variations refers to the relationship between proteins that possess a "common evolutionary  
 15 origin," including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) and homologous proteins from different species (*e.g.*, myosin light chain, etc.) (Reeck et al., 1987, Cell 50:667.). Such proteins (and their encoding genes) have sequence homology, as reflected by their high degree of sequence similarity. However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly,"  
 20 may refer to sequence similarity and not a common evolutionary origin.

Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that may or may not share a common evolutionary origin (*see* Reeck et al., 1987, Cell 50:667).

25 In a specific embodiment, two DNA sequences are "substantially homologous" or "substantially similar" when at least about 50% (preferably at least about 75%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a  
 30 Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, *e.g.*, Sambrook *et al.*, 1989, *supra*.

As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids,

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but do not affect the functional properties of the protein encoded by the DNA sequence. "Substantially similar" also refers to nucleic acid fragments wherein changes in one or more nucleotide bases does not affect the ability of the nucleic acid fragment to mediate alteration of gene expression by antisense or co-suppression technology. "Substantially similar" also refers to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotide bases that do not substantially affect the functional properties of the resulting transcript. It is therefore understood that the invention encompasses more than the specific exemplary sequences. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products.

Moreover, the skilled artisan recognizes that substantially similar sequences encompassed by this invention are also defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS), with the sequences exemplified herein. Substantially similar nucleic acid fragments of the instant invention are those nucleic acid fragments whose DNA sequences are at least 70% identical to the DNA sequence of the nucleic acid fragments reported herein. Preferred substantially nucleic acid fragments of the instant invention are those nucleic acid fragments whose DNA sequences are at least 80% identical to the DNA sequence of the nucleic acid fragments reported herein. More preferred nucleic acid fragments are at least 90% identical to the DNA sequence of the nucleic acid fragments reported herein. Even more preferred are nucleic acid fragments that are at least 95% identical to the DNA sequence of the nucleic acid fragments reported herein.

Two amino acid sequences are "substantially homologous" or "substantially similar" when greater than about 40% of the amino acids are identical, or greater than 60% are similar (functionally identical). Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, *Version 7*, Madison, Wisconsin) pileup program.

The term "corresponding to" is used herein to refer to similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. A nucleic acid or amino acid sequence alignment may include spaces. Thus, the term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

A "substantial portion" of an amino acid or nucleotide sequence comprises enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to putatively

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identify that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., (1993) *J. Mol. Biol.* 215:403-410; see also [www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)). In general, a

5 sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising 20-30 contiguous nucleotides may be used in sequence-

10 *in situ* hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises enough of the sequence to specifically identify and/or isolate a nucleic acid fragment comprising the sequence.

15 The term "percent identity", as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can

20 be readily calculated by known methods, including but not limited to those described in: *Computational Molecular Biology* (Lesk, A. M., ed.) Oxford University Press, New York (1988); *Biocomputing: Informatics and Genome Projects* (Smith, D. W., ed.) Academic Press, New York (1993); *Computer Analysis of Sequence Data, Part I* (Griffin, A. M., and Griffin, H. G., eds.) Humana Press, New Jersey (1994); *Sequence Analysis in Molecular*

25 *Biology* (von Heinje, G., ed.) Academic Press (1987); and *Sequence Analysis Primer* (Gribskov, M. and Devereux, J., eds.) Stockton Press, New York (1991). Preferred methods to determine identity are designed to give the best match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using the

30 Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences may be performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS*. 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for

pairwise alignments using the Clustal method may be selected: KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

The term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" may be commercially available or independently developed. Typical sequence analysis software will include but is not limited to the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI), BLASTP, BLASTN, BLASTX (Altschul et al., *J. Mol. Biol.* 215:403-410 (1990), and DNASTAR (DNASTAR, Inc. 1228 S. Park St. Madison, WI 53715 USA). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters which originally load with the software when first initialized.

"Synthetic genes" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments that are then enzymatically assembled to construct the entire gene. "Chemically synthesized", as related to a sequence of DNA, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available.

#### GENE EXPRESSION MODULATION SYSTEM OF THE INVENTION

Applicants have now shown that separating the transactivation and DNA binding domains by placing them on two different proteins results in greatly reduced background activity in the absence of a ligand and significantly increased activity over background in the presence of a ligand. Applicants' improved gene expression system comprises two chimeric gene expression; the first encoding a DNA binding domain fused to a nuclear receptor polypeptide and the second encoding a transactivation domain fused to a nuclear receptor polypeptide. The interaction of the first protein with the second protein effectively tethers

the DNA binding domain to the transactivation domain. Since the DNA binding and transactivation domains reside on two different molecules, the background activity in the absence of ligand is greatly reduced.

In general, the inducible gene expression modulation system of the invention  
 5 comprises a) a first chimeric gene that is capable of being expressed in a host cell comprising a polynucleotide sequence that encodes a first hybrid polypeptide comprising i) a DNA-binding domain that recognizes a response element associated with a gene whose expression is to be modulated; and ii) a ligand binding domain comprising the ligand binding domain from a nuclear receptor; and b) a second chimeric gene that is capable of being expressed in  
 10 the host cell comprising a polynucleotide sequence that encodes a second hybrid polypeptide comprising: i) a transactivation domain; and ii) a ligand binding domain comprising the ligand binding domain from a nuclear receptor other than ultraspiracle (USP). In a specific embodiment, the gene expression modulation system comprises a DNA binding domain and a transactivation domain from a polypeptide other than a Group H nuclear receptor, an  
 15 ecdysone receptor, a Group B nuclear receptor, a retinoid X receptor, or an ultraspiracle receptor. In another specific embodiment, the ligand binding domains from the first polypeptide and the second polypeptide are different and dimerize.

This two-hybrid system exploits the ability of a pair of interacting proteins to bring the transcription activation domain into a more favorable position relative to the DNA  
 20 binding domain such that when the DNA binding domain binds to the DNA binding site on the gene, the transactivation domain more effectively activates the promoter (see, for example, U.S. Patent No. 5,283,173). This two-hybrid system is a significantly improved inducible gene expression modulation system compared to the two systems disclosed in International Patent Applications PCT/US97/05330 and PCT/US98/14215.

25 The EcR is a member of the nuclear receptor superfamily and classified into subfamily 1, group H (referred to herein as "Group H nuclear receptors"). The members of each group share 40-60% amino acid identity in the E (ligand binding) domain (Laudet et al., A Unified Nomenclature System for the Nuclear Receptor Subfamily, 1999; Cell 97:161-163). In addition to the ecdysone receptor, other members of this nuclear receptor subfamily  
 30 1, group H include: ubiquitous receptor (UR), Orphan receptor 1 (OR-1), steroid hormone nuclear receptor 1 (NER-1), RXR interacting protein-15 (RIP-15), liver x receptor  $\beta$  (LXR $\beta$ ), steroid hormone receptor like protein (RLD-1), liver x receptor (LXR), liver x receptor  $\alpha$  (LXR $\alpha$ ), farnesoid x receptor (FXR), receptor interacting protein 14 (RIP-14), and farnesol receptor (HRR-1).

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Given the close relatedness of ecdysone receptor to other Group H nuclear receptors, Applicants' improved two-hybrid ecdysone receptor-based gene expression system is also expected to work when the ligand binding domains of other Group H nuclear receptors and their respective dimerization partners (Group B nuclear receptors) are used in place of EcR and RXR, respectively. One of ordinary skill in the art is able to make and use a Group H nuclear receptor-based gene expression system based upon Applicants' discovery and disclosure provided herein. Thus, Applicants' invention also provides a two-hybrid Group H nuclear receptor-based gene expression system that is useful in gene modulation for various applications including gene therapy, expression of proteins of interest in host cells, production of transgenic organisms, and cell-based assays.

RXR is a member of the nuclear receptor superfamily and classified into subfamily 2, Group B (referred to herein as "Group B nuclear receptors"). The members of each group share 40-60% amino acid identity in the E (ligand binding) domain (Laudet et al., A Unified Nomenclature System for the Nuclear Receptor Subfamily, 1999; Cell 97:161-163). In addition to the retinoid X receptor, other members of this nuclear receptor subfamily 2, Group B include: H-2 region II binding protein (H-2RIIBP), nuclear receptor co-regulator-1 (RCoR-1), ultraspiracle (USP), 2C1, and chorion factor 1 (CF-1).

The nuclear receptor-based gene expression modulation system of the invention may be either heterodimeric and homodimeric. A functional EcR complex generally refers to a heterodimeric protein complex consisting of two members of the steroid receptor family, an ecdysone receptor protein obtained from various insects, and an ultraspiracle (USP) protein or the vertebrate homolog of USP, retinoid X receptor protein (see Yao, et al. (1993) Nature 366, 476-479; Yao, et al., (1992) Cell 71, 63-72). However, the complex may also be a homodimer as detailed below. The functional ecdysteroid receptor complex may also include additional protein(s) such as immunophilins. Additional members of the steroid receptor family of proteins, known as transcriptional factors (such as DHR38 or *betaFTZ-1*), may also be ligand dependent or independent partners for EcR, USP, and/or RXR. Additionally, other cofactors may be required such as proteins generally known as coactivators (also termed adapters or mediators). These proteins do not bind sequence-specifically to DNA and are not involved in basal transcription. They may exert their effect on transcription activation through various mechanisms, including stimulation of DNA-binding of activators, by affecting chromatin structure, or by mediating activator-initiation complex interactions. Examples of such coactivators include RIP140, TIF1, RAP46/Bag-1, ARA70, SRC-1/NCoA-1, TIF2/GRIP/NCoA-2, ACTR/AIB1/RAC3/pCIP as well as the promiscuous coactivator C

response element B binding protein, CBP/p300 (for review see Glass et al, Curr. Opin. Cell Biol. 9:222-232, 1997). Also, protein cofactors generally known as corepressors (also known as repressors, silencers, or silencing mediators) may be required to effectively inhibit transcriptional activation in the absence of ligand. These corepressors may interact with the

5 unliganded ecdysone receptor to silence the activity at the response element. Current evidence suggests that the binding of ligand changes the conformation of the receptor, which results in release of the corepressor and recruitment of the above described coactivators, thereby abolishing their silencing activity. Examples of corepressors include N-CoR and SMRT (for review, see Horwitz et al. Mol Endocrinol. 10: 1167-1177, 1996). These

10 cofactors may either be endogenous within the cell or organism, or may be added exogenously as transgenes to be expressed in either a regulated or unregulated fashion. Homodimer complexes of the ecdysone receptor protein, USP, or RXR may also be functional under some circumstances.

The ecdysone receptor complex typically includes proteins that are members of the

15 nuclear receptor superfamily wherein all members are generally characterized by the presence of an amino-terminal transactivation domain, a DNA binding domain ("DBD"), and a ligand binding domain ("LBD") separated from the DBD by a hinge region. As used herein, the term "DNA binding domain" comprises a minimal polypeptide sequence of a DNA binding protein, up to the entire length of a DNA binding protein, so long as the DNA

20 binding domain functions to associate with a particular response element. Members of the nuclear receptor superfamily are also characterized by the presence of four or five domains: A/B, C, D, E, and in some members F (see US patent 4,981,784 and Evans, *Science* 240:889-895 (1988)). The "A/B" domain corresponds to the transactivation domain, "C" corresponds to the DNA binding domain, "D" corresponds to the hinge region, and "E" corresponds to

25 the ligand binding domain. Some members of the family may also have another transactivation domain on the carboxy-terminal side of the LBD corresponding to "F".

The DBD is characterized by the presence of two cysteine zinc fingers between which are two amino acid motifs, the P-box and the D-box, which confer specificity for ecdysone response elements. These domains may be either native, modified, or chimeras of

30 different domains of heterologous receptor proteins. The EcR receptor, like a subset of the steroid receptor family, also possesses less well defined regions responsible for heterodimerization properties. Because the domains of nuclear receptors are modular in nature, the LBD, DBD, and transactivation domains may be interchanged.

Gene switch systems are known that incorporate components from the ecdysone

receptor complex. However, in these known systems, whenever EcR is used it is associated with native or modified DNA binding domains and transactivation domains on the same molecule. USP or RXR are typically used as silent partners. We have now shown that when DNA binding domains and transactivation domains are on the same molecule the background activity in the absence of ligand is high and that such activity is dramatically reduced when DNA binding domains and transactivation domains are on different molecules, that is, on each of two partners of a heterodimeric or homodimeric complex. This two-hybrid system also provides improved sensitivity to non-steroidal ligands for example, diacylhydrazines, when compared to steroidal ligands for example, ponasterone A ("PonA") or muristerone A ("MurA"). That is, when compared to steroids, the non-steroidal ligands provide higher activity at a lower concentration. In addition, since transactivation based on EcR gene switches is often cell-line dependent, it is easier to tailor switching system to obtain maximum transactivation capability for each application. Furthermore, this two-hybrid system avoids some side effects due to overexpression of RXR that often occur when unmodified RXR is used as a switching partner. In this two-hybrid system, native DNA binding and transactivation domains of EcR or RXR are eliminated. As a result, these chimeric molecules have less chance of interacting with other steroid hormone receptors present in the cell resulting in reduced side effects.

Specifically, Applicants' invention relates to a gene expression modulation system comprising: a) a first gene expression cassette that is capable of being expressed in a host cell, wherein the first gene expression cassette comprises a polynucleotide that encodes a first polypeptide comprising i) a DNA-binding domain that recognizes a response element associated with a gene whose expression is to be modulated; and ii) a ligand binding domain comprising a ligand binding domain from a nuclear receptor; and b) a second gene expression cassette that is capable of being expressed in the host cell, wherein the second gene expression cassette comprises a polynucleotide sequence that encodes a second polypeptide comprising i) a transactivation domain; and ii) a ligand binding domain comprising a ligand binding domain from a nuclear receptor other than ultraspiracle (USP). In a specific embodiment, the gene expression modulation system comprises a DNA binding domain and a transactivation domain from a polypeptide other than a Group H nuclear receptor, an ecdysone receptor, a Group B nuclear receptor, a retinoid X receptor, or an ultraspiracle receptor. In another specific embodiment, the ligand binding domains from the first polypeptide and the second polypeptide are different and dimerize.

The present invention also relates to a gene expression modulation system according

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to the present invention further comprising c) a third gene expression cassette comprising: i) the response element to which the DNA-binding domain of the first polypeptide binds; ii) a promoter that is activated by the transactivation domain of the second polypeptide; and iii) the gene whose expression is to be modulated. In a specific embodiment, the gene whose  
 5 expression is to be modulated is a homologous gene with respect to the host cell. In another specific embodiment, the gene whose expression is to be modulated is a heterologous gene with respect to the host cell.

In a specific embodiment, the ligand binding domain of the first polypeptide comprises a ligand binding domain selected from the group consisting of an ecdysone  
 10 receptor, ubiquitous receptor (UR), Orphan receptor 1 (OR-1), steroid hormone nuclear receptor 1 (NER-1), RXR interacting protein-15 (RIP-15), liver x receptor  $\beta$  (LXR $\beta$ ), steroid hormone receptor like protein (RLD-1), liver x receptor (LXR), liver x receptor  $\alpha$  (LXR $\alpha$ ), farnesoid x receptor (FXR), receptor interacting protein 14 (RIP-14), and farnesol receptor (HRR-1).

15 In another specific embodiment, the ligand binding domain of the second polypeptide comprises a ligand binding domain selected from the group consisting of a retinoid X receptor, an H-2 region II binding protein (H-2RIIBP), a nuclear receptor co-regulator-1 (RCoR-1), an ultraspiracle (USP), a 2C1, and a chorion factor 1 (CF-1).

In a specific embodiment, the ligand binding domain of the first polypeptide  
 20 comprises a Group H nuclear receptor ligand binding domain. Preferably, the ligand binding domain of the first polypeptide comprises an ecdysone receptor (EcR) ligand binding domain.

In another specific embodiment, ligand binding domain of the second polypeptide comprises a Group B nuclear receptor ligand binding domain. Preferably, the ligand binding domain of the second polypeptide comprises a retinoid X receptor (RXR) ligand binding  
 25 domain.

In a preferred embodiment, when the ligand binding domain is an ecdysone receptor ligand binding domain, the other ligand binding domain ("partner") may be from a vertebrate retinoid X receptor (RXR). The "partner" ligand binding domain may further comprise a truncation mutation, a deletion mutation, a substitution mutation, or another modification.

30 In a specific embodiment, the ligand binding domain of the first polypeptide comprises a Group B nuclear receptor ligand binding domain.

In another specific embodiment, the ligand binding domain of the first polypeptide comprises a ligand binding domain selected from the group consisting of a retinoid X receptor, an H-2 region II binding protein (H-2RIIBP), a nuclear receptor co-regulator-1



(RCoR-1), an ultraspiracle (USP), a 2C1, and a chorion factor 1 (CF-1). Preferably, the ligand binding domain of the first polypeptide comprises a retinoid X receptor ligand binding domain.

In a specific embodiment, the ligand binding domain of the second polypeptide  
5 comprises a Group H nuclear receptor ligand binding domain.

In a specific embodiment, the ligand binding domain of the second polypeptide comprises a ligand binding domain selected from the group consisting of an ecdysone receptor, ubiquitous receptor (UR), Orphan receptor 1 (OR-1), steroid hormone nuclear receptor 1 (NER-1), RXR interacting protein-15 (RIP-15), liver x receptor  $\beta$  (LXR $\beta$ ),  
10 steroid hormone receptor like protein (RLD-1), liver x receptor (LXR), liver x receptor  $\alpha$  (LXR $\alpha$ ), farnesoid x receptor (FXR), receptor interacting protein 14 (RIP-14), and farnesol receptor (HRR-1). Preferably, the ligand binding domain of the second polypeptide comprises an ecdysone receptor ligand binding domain.

In a preferred embodiment, the ligand binding domain of the first polypeptide  
15 comprises an ecdysone receptor ligand binding domain, and the ligand binding domain of the second polypeptide comprises a retinoid X receptor ligand binding domain.

In another preferred embodiment, the ligand binding domain of the first polypeptide is from a retinoid X receptor polypeptide, and the ligand binding domain of the second polypeptide is from an ecdysone receptor polypeptide.

20 Preferably, the ligand binding domain is an EcR or RXR related steroid/thyroid hormone nuclear receptor family member ligand binding domain, or analogs, combinations, or modifications thereof. More preferably, the LBD is from EcR or RXR. Even more preferably, the LBD is from a truncated EcR or RXR. A truncation mutation may be made by any method used in the art, including but not limited to restriction endonuclease  
25 digestion/deletion, PCR-mediated/oligonucleotide-directed deletion, chemical mutagenesis, UV strand breakage, and the like.

Preferably, the EcR is an insect EcR selected from the group consisting of a Lepidopteran EcR, a Dipteran EcR, an Arthropod EcR, a Homopteran EcR and a Hemipteran EcR. More preferably, the EcR for use is a spruce budworm *Choristoneura fumiferana* EcR  
30 ("CfEcR"), a *Tenebrio molitor* EcR ("TmEcR"), a *Manduca sexta* EcR ("MsEcR"), a *Heliothies virescens* EcR ("HvEcR"), a silk moth *Bombyx mori* EcR ("BmEcR"), a fruit fly *Drosophila melanogaster* EcR ("DmEcR"), a mosquito *Aedes aegypti* EcR ("AaEcR"), a blowfly *Lucilia capitata* EcR ("LcEcR"), a Mediterranean fruit fly *Ceratitis capitata* EcR ("CcEcR"), a locust *Locusta migratoria* EcR ("LmEcR"), an aphid *Myzus persicae* EcR

("MpEcR"), a fiddler crab *Uca pugilator* EcR ("UpEcR"), an ixodid tick *Amblyomma americanum* EcR ("AmaEcR"), a white fly *Bemisia argentifolii* EcR ("BaEcR"; Zhang et al., co-pending US provisional patent application filed September 26, 2001, incorporated herein by reference in its entirety), or a green leafhopper *Nephotettix cincticeps* EcR ("NcEcR";

- 5 Palli, co-pending US provisional patent application filed September 26, 2001, incorporated herein by reference in its entirety). Even more preferably, the LBD is from spruce budworm (*Choristoneura fumiferana*) EcR ("CfEcR") or fruit fly *Drosophila melanogaster* EcR ("DmEcR").

- Preferably, the LBD is from a truncated insect EcR. The insect EcR polypeptide
- 10 truncation comprises a deletion of at least 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, or 265 amino acids. More preferably, the insect EcR polypeptide truncation comprises a deletion of at least a partial polypeptide domain. Even more preferably, the
  - 15 insect EcR polypeptide truncation comprises a deletion of at least an entire polypeptide domain. In a specific embodiment, the insect EcR polypeptide truncation comprises a deletion of at least an A/B-domain deletion, a C-domain deletion, a D-domain deletion, an E-domain deletion, an F-domain deletion, an A/B/C-domains deletion, an A/B/1/2-C-domains deletion, an A/B/C/D-domains deletion, an A/B/C/D/F-domains deletion, an A/B/F-domains,
  - 20 and an A/B/C/F-domains deletion. A combination of several complete and/or partial domain deletions may also be performed.

- In a preferred embodiment, the ecdysone receptor ligand binding domain is encoded by a polynucleotide comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:
- 25 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, and SEQ ID NO: 10.

In another preferred embodiment, the ecdysone receptor ligand binding domain comprises a polypeptide sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, and SEQ ID NO: 20.

- 30 Preferably, the vertebrate RXR ligand binding domain is from a human *Homo sapiens*, mouse *Mus musculus*, rat *Rattus norvegicus*, chicken *Gallus gallus*, pig *Sus scrofa domestica*, frog *Xenopus laevis*, zebrafish *Danio rerio*, tunicate *Polyandrocarpa misakiensis*, or jellyfish *Tripedalia cysophora* RXR. More preferably, the vertebrate RXR ligand binding domain is from a mouse *Mus musculus* RXR ("MmRXR") or a human *Homo sapiens* RXR

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("HsRXR"). The vertebrate RXR ligand binding domain is from an RXR $\alpha$ , RXR $\beta$ , or RXR $\gamma$  isoform.

Preferably, the LBD is from a truncated vertebrate RXR. The RXR polypeptide truncation comprises a deletion of at least 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, or 265 amino acids. More preferably, the RXR polypeptide truncation comprises a deletion of at least a partial polypeptide domain. Even more preferably, the RXR polypeptide truncation comprises a deletion of at least an entire polypeptide domain. In a specific embodiment, the RXR polypeptide truncation comprises a deletion of at least an A/B-domain deletion, a C-domain deletion, a D-domain deletion, an E-domain deletion, an F-domain deletion, an A/B/C-domains deletion, an A/B/1/2-C-domains deletion, an A/B/C/D-domains deletion, an A/B/C/D/F-domains deletion, an A/B/F-domains, and an A/B/C/F-domains deletion. A combination of several complete and/or partial domain deletions may also be performed.

In a preferred embodiment, the retinoid X receptor ligand binding domain is encoded by a polynucleotide comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30.

In another preferred embodiment, the retinoid X receptor ligand binding domain comprises a polypeptide sequence selected from the group consisting of SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, and SEQ ID NO: 40.

The DNA binding domain can be any DNA binding domain with a known response element, including synthetic and chimeric DNA binding domains, or analogs, combinations, or modifications thereof. Preferably, the DBD is a GAL4 DBD, a LexA DBD, a transcription factor DBD, a Group H nuclear receptor member DBD, a steroid/thyroid hormone nuclear receptor superfamily member DBD, or a bacterial LacZ DBD. More preferably, the DBD is an EcR DBD [SEQ ID NO: 41 (polynucleotide) or SEQ ID NO: 42 (polypeptide)], a GAL4 DBD [SEQ ID NO: 43 (polynucleotide) or SEQ ID NO: 44 (polypeptide)], or a LexA DBD [(SEQ ID NO: 45 (polynucleotide) or SEQ ID NO: 46 (polypeptide))].

The transactivation domain (abbreviated "AD" or "TA") may be any Group H nuclear receptor member AD, steroid/thyroid hormone nuclear receptor AD, synthetic or

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chimeric AD, polyglutamine AD, basic or acidic amino acid AD, a VP16 AD, a GAL4 AD, an NF- $\kappa$ B AD, a BP64 AD, a B42 acidic activation domain (B42AD), a p65 transactivation domain (p65AD), or an analog, combination, or modification thereof. In a specific embodiment, the AD is a synthetic or chimeric AD, or is obtained from an EcR, a

5 glucocorticoid receptor, VP16, GAL4, NF- $\kappa$ B, or B42 acidic activation domain AD. Preferably, the AD is an EcR AD [SEQ ID NO: 47 (polynucleotide) or SEQ ID NO: 48 (polypeptide)], a VP16 AD [SEQ ID NO: 49 (polynucleotide) or SEQ ID NO: 50 (polypeptide)], a B42 AD [SEQ ID NO: 51 (polynucleotide) or SEQ ID NO: 52 (polypeptide)], or a p65 AD [SEQ ID NO: 53 (polynucleotide) or SEQ ID NO: 54

10 (polypeptide)].

In a specific embodiment, the gene expression cassette encodes a hybrid polypeptide comprising either a) a DNA-binding domain encoded by a polynucleotide comprising a nucleic acid sequence of SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45, or b) a transactivation domain encoded by a polynucleotide comprising a nucleic acid sequence of

15 SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, or SEQ ID NO: 53; and a Group H nuclear receptor ligand binding domain encoded by a polynucleotide according to the invention. Preferably, the Group H nuclear receptor ligand binding domain is an ecdysone receptor ligand binding domain encoded by a polynucleotide according to the invention.

In another specific embodiment, the gene expression cassette encodes a hybrid

20 polypeptide comprising either a) a DNA-binding domain comprising an amino acid sequence of SEQ ID NO: 42, SEQ ID NO: 44, or SEQ ID NO: 46, or b) a transactivation domain comprising an amino acid sequence of SEQ ID NO: 48, EQ ID NO: 50, SEQ ID NO: 52, or SEQ ID NO: 54; and a Group H nuclear receptor ligand binding domain according to the invention. Preferably, the Group H nuclear receptor ligand binding domain is an ecdysone

25 receptor ligand binding domain according to the invention.

The response element ("RE") may be any response element with a known DNA binding domain, or an analog, combination, or modification thereof. A single RE may be employed or multiple REs, either multiple copies of the same RE or two or more different REs, may be used in the present invention. In a specific embodiment, the RE is an RE from

30 GAL4 ("GAL4RE"), LexA, a Group H nuclear receptor RE, a steroid/thyroid hormone nuclear receptor RE, or a synthetic RE that recognizes a synthetic DNA binding domain. Preferably, the RE is an ecdysone response element (EcRE) comprising a polynucleotide sequence of SEQ ID NO: 55, a GAL4RE comprising a polynucleotide sequence of SEQ ID NO: 56, or a LexA RE (operon, "op") comprising a polynucleotide sequence of SEQ ID NO:

57 ("2XLexAopRE").

Preferably, the first hybrid protein is substantially free of a transactivation domain and the second hybrid protein is substantially free of a DNA binding domain. For purposes of this invention, "substantially free" means that the protein in question does not contain a  
5 sufficient sequence of the domain in question to provide activation or binding activity.

The ligands for use in the present invention as described below, when combined with the ligand binding domain of an EcR, USP, RXR, or another polypeptide which in turn are bound to the response element linked to a gene, provide the means for external temporal regulation of expression of the gene. The binding mechanism or the order in which the  
10 various components of this invention bind to each other, that is, for example, ligand to ligand binding domain, DNA-binding domain to response element, transactivation domain to promoter, etc., is not critical. Binding of the ligand to the ligand binding domains of an EcR, USP, RXR, or another protein, enables expression or suppression of the gene. This mechanism does not exclude the potential for ligand binding to EcR, USP, or RXR, and the  
15 resulting formation of active homodimer complexes (e.g. EcR+EcR or USP+USP).

Preferably, one or more of the receptor domains can be varied producing a chimeric gene switch. Typically, one or more of the three domains, DBD, LBD, and transactivation domain, may be chosen from a source different than the source of the other domains so that the chimeric genes and the resulting hybrid proteins are optimized in the chosen host cell or  
20 organism for transactivating activity, complementary binding of the ligand, and recognition of a specific response element. In addition, the response element itself can be modified or substituted with response elements for other DNA binding protein domains such as the GAL-4 protein from yeast (see Sadowski, et al. (1988) *Nature*, 335:563-564) or LexA protein from *E. coli* (see Brent and Ptashne (1985), *Cell*, 43:729-736), or synthetic response elements  
25 specific for targeted interactions with proteins designed, modified, and selected for such specific interactions (see, for example, Kim, et al. (1997), *Proc. Natl. Acad. Sci., USA*, 94:3616-3620) to accommodate chimeric receptors. Another advantage of chimeric systems is that they allow choice of a promoter used to drive the gene expression according to a desired end result. Such double control can be particularly important in areas of gene  
30 therapy, especially when cytotoxic proteins are produced, because both the timing of expression as well as the cells wherein expression occurs can be controlled. When genes, operatively linked to a suitable promoter, are introduced into the cells of the subject, expression of the exogenous genes is controlled by the presence of the system of this invention. Promoters may be constitutively or inducibly regulated or may be tissue-specific

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(that is, expressed only in a particular type of cells) or specific to certain developmental stages of the organism.

#### GENE EXPRESSION CASSETTES OF THE INVENTION

- 5           The novel nuclear receptor-based inducible gene expression system of the invention comprises a novel gene expression cassette that is capable of being expressed in a host cell, wherein the gene expression cassette comprises a polynucleotide encoding a hybrid polypeptide. Thus, Applicants' invention also provides novel gene expression cassettes for use in the gene expression system of the invention.
- 10           Specifically, the present invention provides a gene expression cassette comprising a polynucleotide encoding a hybrid polypeptide. The hybrid polypeptide comprises either 1) a DNA-binding domain that recognizes a response element and a ligand binding domain of a nuclear receptor or 2) a transactivation domain and a ligand binding domain of a nuclear
- 15 receptor, an ecdysone receptor, a Group B nuclear receptor, or a retinoid X receptor. In another specific embodiment, the ligand binding domain and the transactivation domain are from a nuclear receptor other than a Group H nuclear receptor, an EcR, a Group B nuclear receptor, an RXR, or a USP.
- In a specific embodiment, the gene expression cassette encodes a hybrid polypeptide
- 20 comprising a DNA-binding domain that recognizes a response element and an ecdysone receptor ligand binding domain, wherein the DNA binding domain is from a nuclear receptor other than an ecdysone receptor.
- In another specific embodiment, the gene expression cassette encodes a hybrid polypeptide comprising a DNA-binding domain that recognizes a response element and a
- 25 retinoid X receptor ligand binding domain, wherein the DNA binding domain is from a nuclear receptor other than a retinoid X receptor.
- In another specific embodiment, the gene expression cassette encodes a hybrid polypeptide comprising a transactivation domain and an ecdysone receptor ligand binding domain, wherein the transactivation domain is from a nuclear receptor other than an ecdysone
- 30 receptor.
- In another specific embodiment, the gene expression cassette encodes a hybrid polypeptide comprising a transactivation domain and a retinoid X receptor ligand binding domain, wherein the transactivation domain is from a nuclear receptor other than a retinoid X receptor.

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Preferably, the ligand binding domain is an EcR or RXR related steroid/thyroid hormone nuclear receptor family member ligand binding domain, or analogs, combinations, or modifications thereof. More preferably, the LBD is from EcR or RXR. Even more preferably, the LBD is from a truncated EcR or RXR.

- 5 Preferably, the EcR is an insect EcR selected from the group consisting of a Lepidopteran EcR, a Dipteran EcR, an Arthropod EcR, a Homopteran EcR and a Hemipteran EcR. More preferably, the EcR for use is a spruce budworm *Choristoneura fumiferana* EcR ("CfEcR"; Kothapalli et al., 1995 Dev Genet. 17:319-30), a yellow meal worm *Tenebrio molitor* EcR ("TmEcR"; Mouillet et al., 1997, Eur. J. Biochem. 248:856-863), a tobacco
- 10 hornworm *Manduca sexta* EcR ("MsEcR"; Fujiwara et al., 1995, Insect Biochem. Molec. Biol. 25, 845-856), a tobacco budworm *Heliothis virescens* EcR ("HvEcR"; Martinez et al., 1999, *Insect Biochem Mol Biol.* 29:915-30), a golmidge *Chironomus tentans* EcR ("CtEcR"; Imhof et al., 1993, Insect Biochem. Molec. Biol. 23, 115-124), a silkworm *Bombyx mori* EcR ("BmEcR"; Swevers et al., 1995, Insect Biochem. Molec. Biol. 25, 857-866), a squinting
- 15 bush brown *Bicyclus anynana* EcR ("BanEcR"), a buckeye *Junonia coenia* EcR ("JcEcR"), a fruit fly *Drosophila melanogaster* EcR ("DmEcR"; Koelle et al., 1991, Cell 67, 59-77), a yellow fever mosquito *Aedes aegypti* EcR ("AaEcR"; Cho et al., 1995, Insect Biochem. Molec. Biol. 25, 19-27), a blowfly *Lucilia capitata* ("LcEcR"), a sheep blowfly *Lucilia cuprina* EcR ("LucEcR"; Hannan and Hill, 1997, Insect Biochem. Molec. Biol. 27, 479-488),
- 20 a blowfly *Calliphora vicina* EcR ("CvEcR"), a Mediterranean fruit fly *Ceratitis capitata* EcR ("CcEcR"; Verras et al., 1999, *Eur J Biochem.* 265:798-808), a locust *Locusta migratoria* EcR ("LmEcR"; Saleh et al., 1998, *Mol Cell Endocrinol.* 143:91-9), an aphid *Myzus persicae* EcR ("MpEcR"; International Patent Application Publication WO99/36520), a fiddler crab *Celuca pugilator* EcR ("CpEcR"; Chung et al., 1998, *Mol Cell Endocrinol*
- 25 139:209-27), an ixodid tick *Amblyomma americanum* EcR ("AmaEcR"; Guo et al., 1997, Insect Biochem. Molec. Biol. 27, 945-962), a white fly *Bemisia argentifoli* ("BaEcR"; Zhang et al., co-pending US provisional patent application filed September 26, 2001, incorporated herein by reference in its entirety), or a green leafhopper *Nephotetix cincticeps* ("NcEcR"; Palli, co-pending US provisional patent application filed September 26, 2001,
- 30 incorporated herein by reference in its entirety). Even more preferably, the LBD is from spruce budworm (*Choristoneura fumiferana*) EcR ("CfEcR") or fruit fly *Drosophila melanogaster* EcR ("DmEcR").

Preferably, the LBD is from a truncated insect EcR. The insect EcR polypeptide truncation comprises a deletion of at least 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55,

60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, or 265 amino acids. More preferably, the insect EcR polypeptide truncation comprises a deletion of at least a partial polypeptide domain. Even more preferably, the

5 insect EcR polypeptide truncation comprises a deletion of at least an entire polypeptide domain. In a specific embodiment, the insect EcR polypeptide truncation comprises a deletion of at least an A/B-domain deletion, a C-domain deletion, a D-domain deletion, an E-domain deletion, an F-domain deletion, an A/B/C-domains deletion, an A/B/1/2-C-domains deletion, an A/B/C/D-domains deletion, an A/B/C/D/F-domains deletion, an A/B/F-domains,

10 and an A/B/C/F-domains deletion. A combination of several complete and/or partial domain deletions may also be performed.

In a preferred embodiment, the ecdysone receptor ligand binding domain is encoded by a polynucleotide comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:

15 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, and SEQ ID NO: 10.

In another preferred embodiment, the ecdysone receptor ligand binding domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, and SEQ ID NO: 20.

20 Preferably, the RXR polypeptide is a mouse *Mus musculus* RXR ("MmRXR") or a human *Homo sapiens* RXR ("HsRXR"). The RXR polypeptide may be an RXR $_{\alpha}$ , RXR $_{\beta}$ , or RXR $_{\gamma}$  isoform.

Preferably, the LBD is from a truncated RXR. The RXR polypeptide truncation comprises a deletion of at least 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70,

25 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, or 265 amino acids. More preferably, the RXR polypeptide truncation comprises a deletion of at least a partial polypeptide domain. Even more preferably, the RXR polypeptide truncation comprises a deletion of at least an entire polypeptide domain. In a specific embodiment, the

30 RXR polypeptide truncation comprises a deletion of at least an A/B-domain deletion, a C-domain deletion, a D-domain deletion, an E-domain deletion, an F-domain deletion, an A/B/C-domains deletion, an A/B/1/2-C-domains deletion, an A/B/C/D-domains deletion, an A/B/C/D/F-domains deletion, an A/B/F-domains, and an A/B/C/F-domains deletion. A combination of several complete and/or partial domain deletions may also be performed.

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In a preferred embodiment, the retinoid X receptor ligand binding domain is encoded by a polynucleotide comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30.

- 5 In another preferred embodiment, the retinoid X receptor ligand binding domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, and SEQ ID NO: 40.

- The DNA binding domain can be any DNA binding domain with a known response  
10 element, including synthetic and chimeric DNA binding domains, or analogs, combinations, or modifications thereof. Preferably, the DBD is a GAL4 DBD, a LexA DBD, a transcription factor DBD, a Group H nuclear receptor member DBD, a steroid/thyroid hormone nuclear receptor superfamily member DBD, or a bacterial LacZ DBD. More preferably, the DBD is an EcR DBD [SEQ ID NO: 41 (polynucleotide) or SEQ ID NO: 42  
15 (polypeptide)], a GAL4 DBD [SEQ ID NO: 43 (polynucleotide) or SEQ ID NO: 44 (polypeptide)], or a LexA DBD [(SEQ ID NO: 45 (polynucleotide) or SEQ ID NO: 46 (polypeptide))].

- In a preferred embodiment, the gene expression cassette encodes a hybrid polypeptide comprising a DNA-binding domain encoded by a polynucleotide comprising a  
20 nucleic acid sequence selected from the group consisting of an EcR DBD (SEQ ID NO: 41), a GAL4 DBD (SEQ ID NO: 43), and a LexA DBD (SEQ ID NO: 45), and an ecdysone receptor ligand binding domain encoded by a polynucleotide comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID  
25 NO: 9, and SEQ ID NO: 10.

- In another preferred embodiment, the gene expression cassette encodes a hybrid polypeptide comprising a DNA-binding domain comprising a polypeptide sequence selected from the group consisting of an EcR DBD (SEQ ID NO: 42), a GAL4 DBD (SEQ ID NO: 44), and a LexA DBD (SEQ ID NO: 46), and an ecdysone receptor ligand binding domain  
30 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, and SEQ ID NO: 20.

In another preferred embodiment, the gene expression cassette encodes a hybrid polypeptide comprising a DNA-binding domain encoded by a polynucleotide comprising a

nucleic acid sequence selected from the group consisting of an EcR DBD (SEQ ID NO: 41), a GAL4 DBD (SEQ ID NO: 43), and a LexA DBD (SEQ ID NO: 45), and a retinoid X receptor ligand binding domain encoded by a polynucleotide comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30.

In another preferred embodiment, the gene expression cassette encodes a hybrid polypeptide comprising a DNA-binding domain comprising a polypeptide sequence selected from the group consisting of an EcR DBD (SEQ ID NO: 42), a GAL4 DBD (SEQ ID NO: 44), and a LexA DBD (SEQ ID NO: 46), and a retinoid X receptor ligand binding domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, and SEQ ID NO: 40.

The transactivation domain (abbreviated "AD" or "TA") may be any Group H nuclear receptor member AD, steroid/thyroid hormone nuclear receptor AD, synthetic or chimeric AD, polyglutamine AD, basic or acidic amino acid AD, a VP16 AD, a GAL4 AD, an NF- $\kappa$ B AD, a BP64 AD, a B42 acidic activation domain (B42AD), a p65 transactivation domain (p65AD), or an analog, combination, or modification thereof. In a specific embodiment, the AD is a synthetic or chimeric AD, or is obtained from an EcR, a glucocorticoid receptor, VP16, GAL4, NF- $\kappa$ B, or B42 acidic activation domain AD. Preferably, the AD is an EcR AD [SEQ ID NO: 47 (polynucleotide) or SEQ ID NO: 48 (polypeptide)], a VP16 AD [SEQ ID NO: 49 (polynucleotide) or SEQ ID NO: 50 (polypeptide)], a B42 AD [SEQ ID NO: 51 (polynucleotide) or SEQ ID NO: 52 (polypeptide)], or a p65 AD [SEQ ID NO: 53 (polynucleotide) or SEQ ID NO: 54 (polypeptide)].

In a specific embodiment, the gene expression cassette encodes a hybrid polypeptide comprising either a) a DNA-binding domain encoded by a polynucleotide comprising a nucleic acid sequence of SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45, or b) a transactivation domain encoded by a polynucleotide comprising a nucleic acid sequence of SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, or SEQ ID NO: 53; and a Group H nuclear receptor ligand binding domain encoded by a polynucleotide according to the invention. Preferably, the Group H nuclear receptor ligand binding domain is an ecdysone receptor ligand binding domain encoded by a polynucleotide according to the invention.

In another specific embodiment, the gene expression cassette encodes a hybrid

polypeptide comprising either a) a DNA-binding domain comprising an amino acid sequence of SEQ ID NO: 42, SEQ ID NO: 44, or SEQ ID NO: 46, or b) a transactivation domain comprising an amino acid sequence of SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, or SEQ ID NO: 54; and a Group H nuclear receptor ligand binding domain according to the  
 5 invention. Preferably, the Group H nuclear receptor ligand binding domain is an ecdysone receptor ligand binding domain according to the invention.

In a preferred embodiment, the gene expression cassette encodes a hybrid polypeptide comprising a transactivation domain encoded by a polynucleotide comprising a nucleic acid sequence of SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, or SEQ ID NO:  
 10 53, and an ecdysone receptor ligand binding domain encoded by a polynucleotide comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, and SEQ ID NO: 10.

In another preferred embodiment, the gene expression cassette encodes a hybrid  
 15 polypeptide comprising a transactivation domain comprising a polypeptide sequence of SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, or SEQ ID NO: 54, and an ecdysone receptor ligand binding domain comprising a polypeptide sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, and SEQ ID NO: 20.

In another preferred embodiment, the gene expression cassette encodes a hybrid  
 20 polypeptide comprising a transactivation domain encoded by a polynucleotide comprising a nucleic acid sequence of SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, or SEQ ID NO: 53, and a retinoid X receptor ligand binding domain encoded by a polynucleotide comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO:  
 25 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30.

In another preferred embodiment, the gene expression cassette encodes a hybrid polypeptide comprising a transactivation domain comprising a polypeptide sequence of SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, or SEQ ID NO: 54, and a retinoid X receptor  
 30 ligand binding domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, and SEQ ID NO: 40.

The present invention also provides a gene expression cassette comprising: i) a response element comprising a domain recognized by a polypeptide comprising a DNA

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binding domain; ii) a promoter that is activated by a polypeptide comprising a transactivation domain; and iii) a gene whose expression is to be modulated.

The response element ("RE") may be any response element with a known DNA binding domain, or an analog, combination, or modification thereof. A single RE may be employed or multiple REs, either multiple copies of the same RE or two or more different REs, may be used in the present invention. In a specific embodiment, the RE is an RE from GAL4 ("GAL4RE"), LexA, a Group H nuclear receptor RE, a steroid/thyroid hormone nuclear receptor RE, or a synthetic RE that recognizes a synthetic DNA binding domain. Preferably, the RE is an ecdysone response element (EcRE) comprising a polynucleotide sequence of SEQ ID NO: 55, a GAL4RE comprising a polynucleotide sequence of SEQ ID NO: 56, or a LexA RE (operon, "op") comprising a polynucleotide sequence of SEQ ID NO: 57 ("2XLexAopRE").

Preferably, the first hybrid protein is substantially free of a transactivation domain and the second hybrid protein is substantially free of a DNA binding domain. For purposes of this invention, "substantially free" means that the protein in question does not contain a sufficient sequence of the domain in question to provide activation or binding activity.

A steroid/thyroid hormone nuclear receptor DNA binding domain, activation domain or response element according to the invention may be obtained from a steroid/thyroid hormone nuclear receptor selected from the group consisting of thyroid hormone receptor  $\alpha$  (TR $\alpha$ ), thyroid receptor 1 (c-erbA-1), thyroid hormone receptor  $\alpha$  (THRA), thyroid hormone receptor  $\beta$  (TR $\beta$ ), thyroid hormone receptor  $\beta$  (THRB), retinoic acid receptor  $\alpha$  (RAR $\alpha$ ), retinoic acid receptor  $\beta$  (RAR $\beta$ ), hepatoma (HAP), retinoic acid receptor  $\gamma$  (RAR $\gamma$ ), retinoic acid receptor gamma-like (RARD), peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), peroxisome proliferator-activated receptor  $\beta$  (PPAR $\beta$ ), peroxisome proliferator-activator related receptor (NUC-1), peroxisome proliferator-activated receptor  $\delta$  (PPAR $\delta$ ), peroxisome proliferator-activator related receptor (FFAR), peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), orphan receptor encoded by non-encoding strand of thyroid hormone receptor  $\alpha$  (REVERB $\alpha$ ), v-erb A related receptor (EAR-1), v-erb related receptor (EAR-1A),  $\gamma$ ), orphan receptor encoded by non-encoding strand of thyroid hormone receptor  $\beta$  (REVERB $\beta$ ), v-erb related receptor (EAR-1 $\beta$ ), orphan nuclear receptor BD73 (BD73), rev-erbA-related receptor (RVR), zinc finger protein 126 (HZF2), ecdysone-inducible protein E75 (E75), ecdysone-inducible protein E78 (E78), Drosophila receptor 78 (DR-78), retinoid-related orphan receptor  $\alpha$  (ROR $\alpha$ ), retinoid Z receptor  $\alpha$  (RZR $\alpha$ ), retinoid related orphan receptor  $\beta$

- (ROR $\beta$ ), retinoid Z receptor  $\beta$  (RZR $\beta$ ), retinoid-related orphan receptor  $\gamma$  (ROR $\gamma$ ), retinoid Z receptor  $\gamma$  (RZR $\gamma$ ), retinoid-related orphan receptor (TOR), hormone receptor 3 (HR-3), *Drosophila* hormone receptor 3 (DHR-3), myohemerythrin (MHR-3), growth hormone receptor 3 (GHR-3), *C. elegans* nuclear receptor 3 (CNR-3), *C. elegans* hormone receptor 3 (CHR-3), *C. elegans* nuclear receptor 14 (CNR-14), ecdysone receptor (ECR), ubiquitous receptor (UR), orphan nuclear receptor (OR-1), NER-1, receptor-interacting protein 15 (RIP-15), liver X receptor  $\beta$  (LXR $\beta$ ), steroid hormone receptor like protein (RLD-1), liver X receptor (LXR), liver X receptor  $\alpha$  (LXR $\alpha$ ), farnesoid X receptor (FXR), receptor-interacting protein 14 (RIP-14), HRR-1, vitamin D receptor (VDR), orphan nuclear receptor (ONR-1),
- 10 pregnane X receptor (PXR), steroid and xenobiotic receptor (SXR), benzoate X receptor (BXR), nuclear receptor (MB-67), constitutive androstane receptor 1 (CAR-1), constitutive androstane receptor  $\alpha$  (CAR $\alpha$ ), constitutive androstane receptor 2 (CAR-2), constitutive androstane receptor  $\beta$  (CAR $\beta$ ), *Drosophila* hormone receptor 96 (DHR-96), nuclear hormone receptor 1 (NHR-1), hepatocyte nuclear factor 4 (HNF-4), hepatocyte nuclear factor 4G
- 15 (HNF-4G), hepatocyte nuclear factor 4B (HNF-4B), DHNF-4, hepatocyte nuclear factor 4D (HNF-4D), retinoid X receptor  $\alpha$  (RXR $\alpha$ ), retinoid X receptor  $\beta$  (RXR $\beta$ ), H-2 region II binding protein (H-2RIIBP), nuclear receptor co-regulator-1 (RCoR-1), retinoid X receptor  $\gamma$  (RXR $\gamma$ ), Ultraspiracle (USP), 2C1, chorion factor 1 (CF-1), testicular receptor (TR-2), testicular receptor (TR2-11), TR4, TAK-1, *Drosophila* hormone receptor (DHR78), Tailless
- 20 (TLL), tailless homolog (TLX), XTLL, chicken ovalbumin upstream promoter transcription factor I (COUP-TFI), chicken ovalbumin upstream promoter transcription factor A (COUP-TFA), EAR-3, SVP-44, chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), chicken ovalbumin upstream promoter transcription factor B (COUP-TFB), ARP-1, SVP-40, SVP, chicken ovalbumin upstream promoter transcription factor III (COUP-TFIII),
- 25 chicken ovalbumin upstream promoter transcription factor G (COUP-TFG), SVP-46, EAR-2, estrogen receptor  $\alpha$  (ER $\alpha$ ), estrogen receptor  $\beta$  (ER $\beta$ ), estrogen related receptor 1 (ERR1), estrogen related receptor  $\alpha$  (ERR $\alpha$ ), estrogen related receptor 2 (ERR2), estrogen related receptor  $\beta$  (ERR $\beta$ ), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), androgen receptor (AR), nerve growth factor induced gene B
- 30 (NGFI-B), nuclear receptor similar to Nur-77 (TRS), N10, Orphan receptor (NUR-77), Human early response gene (NAK-1), Nurr related factor 1 (NURR-1), a human immediate-early response gene (NOT), regenerating liver nuclear receptor 1 (RNR-1), hematopoietic zinc finger 3 (HZF-3), Nur related protein -1 (TINOR), Nuclear orphan receptor 1 (NOR-1),

- NOR1 related receptor (MINOR), *Drosophila* hormone receptor 38 (DHR-38), *C. elegans* nuclear receptor 8 (CNR-8), C48D5, steroidogenic factor 1 (SF1), endozepine-like peptide (ELP), fushi tarazu factor 1 (FTZ-F1), adrenal 4 binding protein (AD4BP), liver receptor homolog (LRH-1), Ftz-F1-related orphan receptor A (xFFrA), Ftz-F1-related orphan receptor
- 5 B (xFFrB), nuclear receptor related to LRH-1 (FFLR), nuclear receptor related to LRH-1 (PHR), fetoprotein transcriptin factor (FTF), germ cell nuclear factor (GCNFM), retinoid receptor-related testis-associated receptor (RTR), knirps (KNI), knirps related (KNRL), Embryonic gonad (EGON), *Drosophila* gene for ligand dependent nuclear receptor (EAGLE), nuclear receptor similar to trithorax (ODR7), Trithorax, dosage sensitive sex
- 10 reversal adrenal hypoplasia congenita critical region chromosome X gene (DAX-1), adrenal hypoplasia congenita and hypogonadotropic hypogonadism (AHCH), and short heterodimer partner (SHP).

- For purposes of this invention, Group H nuclear receptors, EcR and RXR also include synthetic and chimeric Group H nuclear receptors, EcR and RXR and their
- 15 homologs.

- Genes of interest for use in Applicants' gene expression cassettes may be endogenous genes or heterologous genes. Nucleic acid or amino acid sequence information for a desired gene or protein can be located in one of many public access databases, for example, GENBANK, EMBL, Swiss-Prot, and PIR, or in many biology related journal
- 20 publications. Thus, those skilled in the art have access to nucleic acid sequence information for virtually all known genes. Such information can then be used to construct the desired constructs for the insertion of the gene of interest within the gene expression cassettes used in Applicants' methods described herein.

- Examples of genes of interest for use in Applicants' gene expression cassettes
- 25 include, but are not limited to: genes encoding therapeutically desirable polypeptides or products that may be used to treat a condition, a disease, a disorder, a dysfunction, a genetic defect, such as monoclonal antibodies, enzymes, proteases, cytokines, interferons, insulin, erthropoietin, clotting factors, other blood factors or components, viral vectors for gene therapy, virus for vaccines, targets for drug discovery, functional genomics, and proteomics
- 30 analyses and applications, and the like.

#### POLYNUCLEOTIDES OF THE INVENTION

The novel two-hybrid inducible gene expression system of the invention comprises a gene expression cassette comprising a polynucleotide that encodes a nuclear receptor ligand

binding domain. In a specific embodiment, the ligand binding domain is from a Group H nuclear receptor, an ecdysone receptor, a Group B nuclear receptor, or a retinoid X receptor.

In a preferred embodiment, the two-hybrid inducible gene expression system is a two-hybrid ecdysone receptor-based inducible gene expression system of the invention comprising a  
 5 gene expression cassette comprising a polynucleotide that encodes a ligand binding domain from an EcR or RXR polypeptide. Preferably, the polynucleotide encoding the ligand binding domain from an EcR or RXR polypeptide, is a polynucleotide encoding an EcR or RXR polypeptide comprising a truncation mutation. These gene expression cassettes and the polynucleotides they comprise are useful as components of a nuclear receptor-based gene  
 10 expression system to modulate the expression of a gene within a host cell.

Thus, the present invention also relates to a polynucleotide that encodes a Group H nuclear receptor polypeptide comprising a truncation mutation, an ecdysone receptor polypeptide comprising a truncation mutation, a Group B nuclear receptor polypeptide comprising a truncation mutation, or a retinoid X receptor polypeptide comprising a  
 15 truncation mutation. The present invention also relates to a polynucleotide that encodes an EcR or RXR polypeptide comprising a truncation mutation. Specifically, the present invention relates to an isolated polynucleotide encoding an EcR or RXR polypeptide comprising a truncation mutation that affects ligand binding activity or ligand sensitivity.

Preferably, the truncation mutation results in a polynucleotide that encodes a  
 20 truncated EcR polypeptide or a truncated RXR polypeptide comprising a deletion of at least 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, or 265 amino acids. More preferably, the EcR or RXR polypeptide truncation comprises a deletion of at least a partial  
 25 polypeptide domain. Even more preferably, the EcR or RXR polypeptide truncation comprises a deletion of at least an entire polypeptide domain. In a specific embodiment, the EcR or RXR polypeptide truncation comprises a deletion of at least an A/B-domain deletion, a C-domain deletion, a D-domain deletion, an E-domain deletion, an F-domain deletion, an A/B/C-domains deletion, an A/B/1/2-C-domains deletion, an A/B/C/D-domains deletion, an  
 30 A/B/C/D/F-domains deletion, an A/B/F-domains, and an A/B/C/F-domains deletion. A combination of several complete and/or partial domain deletions may also be performed.

In a specific embodiment, the EcR polynucleotide according to the invention comprises a polynucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO:

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7, SEQ ID NO: 8, SEQ ID NO: 9, and SEQ ID NO: 10. In a specific embodiment, the polynucleotide according to the invention encodes a ecdysone receptor polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 11 (CfEcR-CDEF), SEQ ID NO: 12 (CfEcR-1/2CDEF, which comprises the last 33 carboxy-terminal amino acids of C domain), SEQ ID NO: 13 (CfEcR-DEF), SEQ ID NO: 14 (CfEcR-EF), SEQ ID NO: 15 (CfEcR-DE), SEQ ID NO: 16 (DmEcR-CDEF), SEQ ID NO: 17 (DmEcR-1/2CDEF), SEQ ID NO: 18 (DmEcR-DEF), SEQ ID NO: 19 (DmEcR-EF), and SEQ ID NO: 20 (DmEcR-DE).

In another specific embodiment, the RXR polynucleotide according to the invention comprises a polynucleotide sequence selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30. In another specific embodiment, the polynucleotide according to the invention encodes a truncated RXR polypeptide comprising an amino acid sequence consisting of SEQ ID NO: 31 (MmRXR-CDEF), SEQ ID NO: 32 (MmRXR-DEF), SEQ ID NO: 33 (MmRXR-EF), SEQ ID NO: 34 (MmRXR-truncatedEF), SEQ ID NO: 35 (MmRXR-E), SEQ ID NO: 36 (HsRXR-CDEF), SEQ ID NO: 37 (HsRXR-DEF), SEQ ID NO: 38 (HsRXR-EF), SEQ ID NO: 39 (HsRXR-truncated EF), and SEQ ID NO: 40 (HsRXR-E).

In particular, the present invention relates to an isolated polynucleotide encoding an EcR or RXR polypeptide comprising a truncation mutation, wherein the mutation reduces ligand binding activity or ligand sensitivity of the EcR or RXR polypeptide. In a specific embodiment, the present invention relates to an isolated polynucleotide encoding an EcR or RXR polypeptide comprising a truncation mutation that reduces steroid binding activity or steroid sensitivity of the EcR or RXR polypeptide. In a preferred embodiment, the present invention relates to an isolated polynucleotide encoding an EcR polypeptide comprising a truncation mutation that reduces steroid binding activity or steroid sensitivity of the EcR polypeptide, wherein the polynucleotide comprises a nucleic acid sequence of SEQ ID NO: 3 (CfEcR-DEF), SEQ ID NO: 4 (CfEcR-EF), SEQ ID NO: 8 (DmEcR-DEF), or SEQ ID NO: 9 (DmEcR-EF). In another specific embodiment, the present invention relates to an isolated polynucleotide encoding an EcR or RXR polypeptide comprising a truncation mutation that reduces non-steroid binding activity or non-steroid sensitivity of the EcR or RXR polypeptide. In a preferred embodiment, the present invention relates to an isolated polynucleotide encoding an EcR polypeptide comprising a truncation mutation that reduces non-steroid binding activity or non-steroid sensitivity of the EcR polypeptide, wherein the



polynucleotide comprises a nucleic acid sequence of SEQ ID NO: 4 (CfEcR-EF) or SEQ ID NO: 9 (DmEcR-EF).

The present invention also relates to an isolated polynucleotide encoding an EcR or RXR polypeptide comprising a truncation mutation, wherein the mutation enhances ligand binding activity or ligand sensitivity of the EcR or RXR polypeptide. In a specific embodiment, the present invention relates to an isolated polynucleotide encoding an EcR or RXR polypeptide comprising a truncation mutation that enhances steroid binding activity or steroid sensitivity of the EcR or RXR polypeptide. In another specific embodiment, the present invention relates to an isolated polynucleotide encoding an EcR or RXR polypeptide comprising a truncation mutation that enhances non-steroid binding activity or non-steroid sensitivity of the EcR or RXR polypeptide. In a preferred embodiment, the present invention relates to an isolated polynucleotide encoding an EcR polypeptide comprising a truncation mutation that enhances non-steroid binding activity or non-steroid sensitivity of the EcR polypeptide, wherein the polynucleotide comprises a nucleic acid sequence of SEQ ID NO: 3 (CfEcR-DEF) or SEQ ID NO: 8 (DmEcR-DEF).

The present invention also relates to an isolated polynucleotide encoding a retinoid X receptor polypeptide comprising a truncation mutation that increases ligand sensitivity of a heterodimer comprising the mutated retinoid X receptor polypeptide and a dimerization partner. Preferably, the isolated polynucleotide encoding a retinoid X receptor polypeptide comprising a truncation mutation that increases ligand sensitivity of a heterodimer comprises a polynucleotide sequence selected from the group consisting of SEQ ID NO: 23 (MmRXR-EF), SEQ ID NO: 24 (MmRXR-truncatedEF), SEQ ID NO: 28 (HsRXR-EF), or SEQ ID NO: 29 (HsRXR-truncated EF). In a specific embodiment, the dimerization partner is an ecdysone receptor polypeptide. Preferably, the dimerization partner is a truncated EcR polypeptide. More preferably, the dimerization partner is an EcR polypeptide in which domains A/B/C have been deleted. Even more preferably, the dimerization partner is an EcR polypeptide comprising an amino acid sequence of SEQ ID NO: 13 (CfEcR-DEF) or SEQ ID NO: 18 (DmEcR-DEF).

In addition, the present invention relates to an expression vector comprising a polynucleotide according to the invention, operatively linked to a transcription regulatory element. Preferably, the polynucleotide encoding a ligand binding domain is operatively linked with an expression control sequence permitting expression of the ligand binding domain in an expression competent host cell. The expression control sequence may comprise a promoter that is functional in the host cell in which expression is desired. The vector may

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be a plasmid DNA molecule or a viral vector. Preferred viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, and vaccinia virus. The invention further relates to a replication defective recombinant virus comprising in its genome, the polynucleotide encoding a ligand binding domain as described above. Thus, the present invention also relates to an isolated host cell comprising such an expression vector, wherein the transcription regulatory element is operative in the host cell.

The present invention also relates to an isolated polypeptide encoded by a polynucleotide according to the invention.

## 10 POLYPEPTIDES OF THE INVENTION

The novel two-hybrid-based inducible gene expression system of the invention comprises at least one gene expression cassette comprising a polynucleotide that encodes a polypeptide comprising a nuclear receptor ligand binding domain. In a specific embodiment, the nuclear receptor ligand binding domain is from a Group H nuclear receptor, an ecdysone receptor, a Group B nuclear receptor, or a retinoid X receptor. In a preferred embodiment, the two-hybrid inducible gene expression system is a two-hybrid ecdysone receptor-based inducible gene expression system of the invention comprising a gene expression cassette comprising a polynucleotide that encodes a ligand binding domain from an EcR or RXR polypeptide. Preferably, the polynucleotide encoding the ligand binding domain from an EcR or RXR polypeptide, is a polynucleotide encoding an EcR or RXR polypeptide comprising a truncation mutation. These gene expression cassettes and polynucleotides and the polypeptides they encode are useful as components of a nuclear receptor-based gene expression system to modulate the expression of a gene within a host cell. Thus, the present invention also provides an isolated polypeptide comprising a nuclear receptor ligand binding domain according to the invention.

Thus, the present invention also relates to an isolated truncated Group H nuclear receptor, truncated EcR, truncated Group B nuclear receptor, or truncated RXR polypeptide encoded by a polynucleotide or a gene expression cassette according to the invention. Specifically, the present invention relates to an isolated truncated EcR or RXR polypeptide comprising a truncation mutation that affects ligand binding activity or ligand sensitivity encoded by a polynucleotide according to the invention.

The present invention also relates to an isolated truncated Group H nuclear receptor, truncated EcR, truncated Group B nuclear receptor, or truncated RXR polypeptide comprising a truncation mutation. Specifically, the present invention relates to an isolated

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EcR or RXR polypeptide comprising a truncation mutation that affects ligand binding activity or ligand sensitivity.

Preferably, the truncation mutation results in a truncated EcR polypeptide or a truncated RXR polypeptide comprising a deletion of at least 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, or 265 amino acids. More preferably, the EcR or RXR polypeptide truncation comprises a deletion of at least a partial polypeptide domain. Even more preferably, the EcR or RXR polypeptide truncation comprises a deletion of at least an entire polypeptide domain. In a specific embodiment, the EcR or RXR polypeptide truncation comprises a deletion of at least an A/B-domain deletion, a C-domain deletion, a D-domain deletion, an E-domain deletion, an F-domain deletion, an A/B/C-domains deletion, an A/B/1/2-C-domains deletion, an A/B/C/D-domains deletion, an A/B/C/D/F-domains deletion, an A/B/F-domains, and an A/B/C/F-domains deletion. A combination of several complete and/or partial domain deletions may also be performed.

In a preferred embodiment, the isolated truncated ecdysone receptor polypeptide is encoded by a polynucleotide comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO: 1 (CfEcR-CDEF), SEQ ID NO: 2 (CfEcR-1/2CDEF), SEQ ID NO: 3 (CfEcR-DEF), SEQ ID NO: 4 (CfEcR-EF), SEQ ID NO: 5 (CfEcR-DE), SEQ ID NO: 6 (DmEcR-CDEF), SEQ ID NO: 7 (DmEcR-1/2CDEF), SEQ ID NO: 8 (DmEcR-DEF), SEQ ID NO: 9 (DmEcR-EF), and SEQ ID NO: 10 (DmEcR-DE). In another preferred embodiment, the isolated truncated ecdysone receptor polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 11 (CfEcR-CDEF), SEQ ID NO: 12 (CfEcR-1/2CDEF), SEQ ID NO: 13 (CfEcR-DEF), SEQ ID NO: 14 (CfEcR-EF), SEQ ID NO: 15 (CfEcR-DE), SEQ ID NO: 16 (DmEcR-CDEF), SEQ ID NO: 17 (DmEcR-1/2CDEF), SEQ ID NO: 18 (DmEcR-DEF), SEQ ID NO: 19 (DmEcR-EF), and SEQ ID NO: 20 (DmEcR-DE).

In a preferred embodiment, the isolated truncated RXR polypeptide is encoded by a polynucleotide comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO: 21 (MmRXR-CDEF), SEQ ID NO: 22 (MmRXR-DEF), SEQ ID NO: 23 (MmRXR-EF), SEQ ID NO: 24 (MmRXR-truncatedEF), SEQ ID NO: 25 (MmRXR-E), SEQ ID NO: 26 (HsRXR-CDEF), SEQ ID NO: 27 (HsRXR-DEF), SEQ ID NO: 28 (HsRXR-EF), SEQ ID NO: 29 (HsRXR-truncatedEF) and SEQ ID NO: 30 (HsRXR-E). In another preferred embodiment, the isolated truncated RXR polypeptide comprises an amino acid

sequence selected from the group consisting of SEQ ID NO: 31 (MmRXR-CDEF), SEQ ID NO: 32 (MmRXR-DEF), SEQ ID NO: 33 (MmRXR-EF), SEQ ID NO: 34 (MmRXR-truncatedEF), SEQ ID NO: 35 (MmRXR-E), SEQ ID NO: 36 (HsRXR-CDEF), SEQ ID NO: 37 (HsRXR-DEF), SEQ ID NO: 38 (HsRXR-EF), SEQ ID NO: 39 (HsRXR-truncatedEF),  
 5 and SEQ ID NO: 40 (HsRXR-E).

The present invention relates to an isolated EcR or RXR polypeptide comprising a truncation mutation that reduces ligand binding activity or ligand sensitivity of the EcR or RXR polypeptide, wherein the polypeptide is encoded by a polynucleotide comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO: 1 (CfEcR-  
 10 CDEF), SEQ ID NO: 2 (CfEcR-1/2CDEF), SEQ ID NO: 3 (CfEcR-DEF), SEQ ID NO: 4 (CfEcR-EF), SEQ ID NO: 5 (CfEcR-DE), SEQ ID NO: 6 (DmEcR-CDEF), SEQ ID NO: 7 (DmEcR-1/2CDEF), SEQ ID NO: 8 (DmEcR-DEF), SEQ ID NO: 9 (DmEcR-EF), SEQ ID NO: 10 (DmEcR-DE), SEQ ID NO: 21 (MmRXR-CDEF), SEQ ID NO: 22 (MmRXR-DEF), SEQ ID NO: 23 (MmRXR-EF), SEQ ID NO: 24 (MmRXR-truncatedEF), SEQ ID NO: 25  
 15 (MmRXR-E), SEQ ID NO: 26 (HsRXR-CDEF), SEQ ID NO: 27 (HsRXR-DEF), SEQ ID NO: 28 (HsRXR-EF), SEQ ID NO: 29 (HsRXR-truncatedEF), and SEQ ID NO: 30 (HsRXR-E).

Thus, the present invention relates to an isolated truncated EcR or RXR polypeptide comprising a truncation mutation that reduces ligand binding activity or ligand sensitivity of  
 20 the EcR or RXR polypeptide, wherein the polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 11 (CfEcR-CDEF), SEQ ID NO: 12 (CfEcR-1/2CDEF), SEQ ID NO: 13 (CfEcR-DEF), SEQ ID NO: 14 (CfEcR-EF), SEQ ID NO: 15 (CfEcR-DE), SEQ ID NO: 16 (DmEcR-CDEF), SEQ ID NO: 17 (DmEcR-1/2CDEF), SEQ ID NO: 18 (DmEcR-DEF), SEQ ID NO: 19 (DmEcR-EF), SEQ ID NO: 20 (DmEcR-  
 25 DE), SEQ ID NO: 31 (MmRXR-CDEF), SEQ ID NO: 32 (MmRXR-DEF), SEQ ID NO: 33 (MmRXR-EF), SEQ ID NO: 34 (MmRXR-truncatedEF), SEQ ID NO: 35 (MmRXR-E), SEQ ID NO: 36 (HsRXR-CDEF), SEQ ID NO: 37 (HsRXR-DEF), SEQ ID NO: 38 (HsRXR-EF), SEQ ID NO: 39 (HsRXR-truncatedEF), and SEQ ID NO: 40 (HsRXR-E).

In a specific embodiment, the present invention relates to an isolated EcR or RXR  
 30 polypeptide comprising a truncation mutation that reduces steroid binding activity or steroid sensitivity of the EcR or RXR polypeptide. In a preferred embodiment, the present invention relates to an isolated EcR polypeptide comprising a truncation mutation that reduces steroid binding activity or steroid sensitivity of the EcR polypeptide, wherein the EcR polypeptide is encoded by a polynucleotide comprising a nucleic acid sequence of SEQ ID NO: 3 (CfEcR-

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DEF), SEQ ID NO: 4 (CfEcR-EF), SEQ ID NO: 8 (DmEcR-DEF), or SEQ ID NO: 9 (DmEcR-EF). Accordingly, the present invention also relates to an isolated truncated EcR or RXR polypeptide comprising a truncation mutation that reduces steroid binding activity or steroid sensitivity of the EcR or RXR polypeptide. In a preferred embodiment, the present invention relates to an isolated EcR polypeptide comprising a truncation mutation that reduces steroid binding activity or steroid sensitivity of the EcR polypeptide, wherein the EcR polypeptide comprises an amino acid sequence of SEQ ID NO: 13 (CfEcR-DEF), SEQ ID NO: 14 (CfEcR-EF), SEQ ID NO: 18 (DmEcR-DEF), or SEQ ID NO: 19 (DmEcR-EF).

In another specific embodiment, the present invention relates to an isolated EcR or RXR polypeptide comprising a truncation mutation that reduces non-steroid binding activity or non-steroid sensitivity of the EcR or RXR polypeptide. In a preferred embodiment, the present invention relates to an isolated EcR polypeptide comprising a truncation mutation that reduces non-steroid binding activity or non-steroid sensitivity of the EcR polypeptide, wherein the EcR polypeptide is encoded by a polynucleotide comprising a nucleic acid sequence of SEQ ID NO: 4 (CfEcR-EF) or SEQ ID NO: 9 (DmEcR-EF). Accordingly, the present invention also relates to an isolated truncated EcR or RXR polypeptide comprising a truncation mutation that reduces non-steroid binding activity or steroid sensitivity of the EcR or RXR polypeptide. In a preferred embodiment, the present invention relates to an isolated EcR polypeptide comprising a truncation mutation that reduces non-steroid binding activity or non-steroid sensitivity of the EcR polypeptide, wherein the EcR polypeptide comprises an amino acid sequence of SEQ ID NO: 14 (CfEcR-EF) or SEQ ID NO: 19 (DmEcR-EF).

In particular, the present invention relates to an isolated EcR or RXR polypeptide comprising a truncation mutation that enhances ligand binding activity or ligand sensitivity of the EcR or RXR polypeptide, wherein the polypeptide is encoded by a polynucleotide comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO: 1 (CfEcR-CDEF), SEQ ID NO: 2 (CfEcR-1/2CDEF), SEQ ID NO: 3 (CfEcR-DEF), SEQ ID NO: 4 (CfEcR-EF), SEQ ID NO: 5 (CfEcR-DE), SEQ ID NO: 6 (DmEcR-CDEF), SEQ ID NO: 7 (DmEcR-1/2CDEF), SEQ ID NO: 8 (DmEcR-DEF), SEQ ID NO: 9 (DmEcR-EF), SEQ ID NO: 10 (DmEcR-DE), SEQ ID NO: 21 (MmRXR-CDEF), SEQ ID NO: 22 (MmRXR-DEF), SEQ ID NO: 23 (MmRXR-EF), SEQ ID NO: 24 (MmRXR-truncatedEF), SEQ ID NO: 25 (MmRXR-E), SEQ ID NO: 26 (HsRXR-CDEF), SEQ ID NO: 27 (HsRXR-DEF), SEQ ID NO: 28 (HsRXR-EF), SEQ ID NO: 29 (HsRXR-truncated EF), and SEQ ID NO: 30 (HsRXR-E).

The present invention relates to an isolated EcR or RXR polypeptide comprising a

truncation mutation that enhances ligand binding activity or ligand sensitivity of the EcR or RXR polypeptide, wherein the polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 11 (CfEcR-CDEF), SEQ ID NO: 12 (CfEcR-1/2CDEF), SEQ ID NO: 13 (CfEcR-DEF), SEQ ID NO: 14 (CfEcR-EF), SEQ ID NO: 15 (CfEcR-DE),

5 SEQ ID NO: 16 (DmEcR-CDEF), SEQ ID NO: 17 (DmEcR-1/2CDEF), SEQ ID NO: 18 (DmEcR-DEF), SEQ ID NO: 19 (DmEcR-EF), SEQ ID NO: 20 (DmEcR-DE), SEQ ID NO: 31 (MmRXR-CDEF), SEQ ID NO: 32 (MmRXR-DEF), SEQ ID NO: 33 (MmRXR-EF), SEQ ID NO: 34 (MmRXR-truncatedEF), SEQ ID NO: 35 (MmRXR-E), SEQ ID NO: 36 (HsRXR-CDEF), SEQ ID NO: 37 (HsRXR-DEF), SEQ ID NO: 39 (HsRXR-EF), SEQ ID NO: 39

10 (HsRXR-truncatedEF), and SEQ ID NO: 40 (HsRXR-E).

The present invention relates to an isolated EcR or RXR polypeptide comprising a truncation mutation that enhances ligand binding activity or ligand sensitivity of the EcR or RXR polypeptide. In a specific embodiment, the present invention relates to an isolated EcR or RXR polypeptide comprising a truncation mutation that enhances steroid binding activity

15 or steroid sensitivity of the EcR or RXR polypeptide. Accordingly, the present invention also relates to an isolated EcR or RXR polypeptide comprising a truncation mutation that enhances steroid binding activity or steroid sensitivity of the EcR or RXR polypeptide.

In another specific embodiment, the present invention relates to an isolated EcR or RXR polypeptide comprising a truncation mutation that enhances non-steroid binding

20 activity or non-steroid sensitivity of the EcR or RXR polypeptide. In a preferred embodiment, the present invention relates to an isolated EcR polypeptide comprising a truncation mutation that enhances non-steroid binding activity or non-steroid sensitivity of the EcR polypeptide, wherein the EcR polypeptide is encoded by a polynucleotide comprising a nucleic acid sequence of SEQ ID NO: 3 (CfEcR-DEF) or SEQ ID NO: 8 (DmEcR-DEF). Accordingly,

25 the present invention also relates to an isolated EcR or RXR polypeptide comprising a truncation mutation that enhances non-steroid binding activity or steroid sensitivity of the EcR or RXR polypeptide. In a preferred embodiment, the present invention relates to an isolated EcR polypeptide comprising a truncation mutation that enhances non-steroid binding activity or non-steroid sensitivity of the EcR polypeptide, wherein the EcR polynucleotide

30 comprises an amino acid sequence of SEQ ID NO: 13 (CfEcR-DEF) or SEQ ID NO: 18 (DmEcR-DEF).

The present invention also relates to an isolated retinoid X receptor polypeptide comprising a truncation mutation that increases ligand sensitivity of a heterodimer comprising the mutated retinoid X receptor polypeptide and a dimerization partner.

Preferably, the isolated retinoid X receptor polypeptide comprising a truncation mutation that increases ligand sensitivity of a heterodimer is encoded by a polynucleotide comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 23 (MmRXR-EF), SEQ ID NO: 24 (MmRXR-truncatedEF), SEQ ID NO: 28 (HsRXR-EF), or SEQ ID NO: 29 (HsRXR-truncatedEF). More preferably, the isolated polynucleotide encoding a retinoid X receptor polypeptide comprising a truncation mutation that increases ligand sensitivity of a heterodimer comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 33 (MmRXR-EF), SEQ ID NO: 34 (MmRXR-truncatedEF), SEQ ID NO: 38 (HsRXR-EF), or SEQ ID NO: 39 (HsRXR-truncatedEF).

10 In a specific embodiment, the dimerization partner is an ecdysone receptor polypeptide. Preferably, the dimerization partner is a truncated EcR polypeptide. More preferably, the dimerization partner is an EcR polypeptide in which domains A/B/C have been deleted. Even more preferably, the dimerization partner is an EcR polypeptide comprising an amino acid sequence of SEQ ID NO: 13 (CfEcR-DEF) or SEQ ID NO: 18 (DmEcR-DEF).

The present invention also relates to compositions comprising an isolated polypeptide according to the invention.

#### Compositions

20 The present invention also relates to compositions comprising the isolated polynucleotides or polypeptides according to the invention. Such compositions may comprise a polypeptide or a polynucleotide encoding a polypeptide, as defined above, and an acceptable carrier or vehicle. The compositions of the invention are particularly suitable for formulation of biological material for use in a gene expression modulation system according to the invention. Thus, in a preferred embodiment, the composition comprises a polynucleotide encoding a polypeptide according to the invention. In another preferred embodiment, the composition comprises a polypeptide according to the invention.

The phrase "acceptable" refers to molecular entities and compositions that are physiologically tolerable to the cell or organism when administered. The term "carrier" 30 refers to a diluent, adjuvant, excipient, or vehicle with which the composition is administered. Such carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Examples of acceptable carriers are saline, buffered saline, isotonic saline (e.g., monosodium or disodium phosphate, sodium, potassium, calcium or magnesium

chloride, or mixtures of such salts), Ringer's solution, dextrose, water, sterile water, glycerol, ethanol, and combinations thereof. 1,3-butanediol and sterile fixed oils are conveniently employed as solvents or suspending media. Any bland fixed oil can be employed including synthetic mono- or di-glycerides. Fatty acids such as oleic acid also find use in the

5 preparation of injectables. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Pharmaceutical compositions of the invention may be formulated for the purpose of topical, oral, parenteral, intranasal, intravenous, intramuscular,

10 subcutaneous, intraocular, and the like, administration.

Preferably, the compositions comprise an acceptable vehicle for an injectable formulation. This vehicle can be, in particular, a sterile, isotonic saline solution (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride, and the like, or mixtures of such salts), or dry, in particular lyophilized, compositions which, on addition, as

15 appropriate, of sterilized water or of physiological saline, enable injectable solutions to be formed. The preferred sterile injectable preparations can be a solution or suspension in a nontoxic parenterally acceptable solvent or diluent.

In yet another embodiment, a composition comprising a polypeptide according to the invention, or polynucleotide encoding the polypeptide, can be delivered in a controlled

20 release system. For example, the polynucleotide or polypeptide may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. Other controlled release systems are discussed in the review by Langer [*Science* 249:1527-1533 (1990)].

## 25 METHOD OF MODULATING GENE EXPRESSION OF THE INVENTION

Applicants' invention also relates to methods of modulating gene expression in a host cell using a gene expression modulation system according to the invention. Specifically, Applicants' invention provides a method of modulating the expression of a gene in a host cell comprising the steps of: a) introducing into the host cell a gene expression modulation

30 system according to the invention; and b) introducing into the host cell a ligand; wherein the gene to be modulated is a component of a gene expression cassette comprising: i) a response element comprising a domain recognized by the DNA binding domain of the gene expression system; ii) a promoter that is activated by the transactivation domain of the gene expression system; and iii) a gene whose expression is to be modulated, whereby upon introduction of

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the ligand into the host cell, expression of the gene is modulated.

The invention also provides a method of modulating the expression of a gene in a host cell comprising the steps of: a) introducing into the host cell a gene expression modulation system according to the invention; b) introducing into the host cell a gene expression cassette according to the invention, wherein the gene expression cassette comprises i) a response element comprising a domain recognized by the DNA binding domain from the gene expression system; ii) a promoter that is activated by the transactivation domain of the gene expression system; and iii) a gene whose expression is to be modulated; and c) introducing into the host cell a ligand; whereby upon introduction of the ligand into the host cell, expression of the gene is modulated.

Applicants' invention also provides a method of modulating the expression of a gene in a host cell comprising a gene expression cassette comprising a response element comprising a domain to which the DNA binding domain from the first hybrid polypeptide of the gene expression modulation system binds; a promoter that is activated by the transactivation domain of the second hybrid polypeptide of the gene expression modulation system; and a gene whose expression is to be modulated; wherein the method comprises the steps of: a) introducing into the host cell a gene expression modulation system according to the invention; and b) introducing into the host cell a ligand; whereby upon introduction of the ligand into the host, expression of the gene is modulated.

Genes of interest for expression in a host cell using Applicants' methods may be endogenous genes or heterologous genes. Nucleic acid or amino acid sequence information for a desired gene or protein can be located in one of many public access databases, for example, GENBANK, EMBL, Swiss-Prot, and PIR, or in many biology related journal publications. Thus, those skilled in the art have access to nucleic acid sequence information for virtually all known genes. Such information can then be used to construct the desired constructs for the insertion of the gene of interest within the gene expression cassettes used in Applicants' methods described herein.

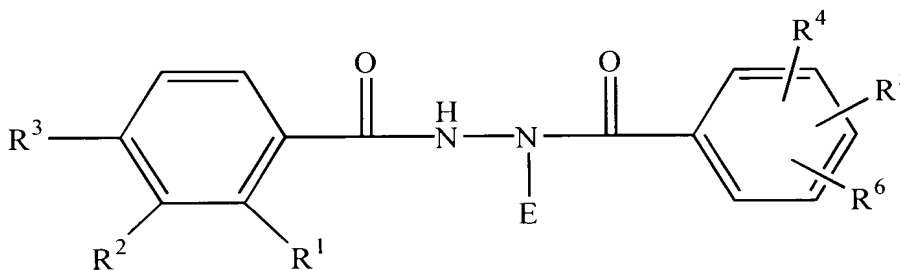
Examples of genes of interest for expression in a host cell using Applicants' methods include, but are not limited to: antigens produced in plants as vaccines, enzymes like alpha-amylase, phytase, glucanase, and xylanase, genes for resistance against insects, nematodes, fungi, bacteria, viruses, and abiotic stresses, nutraceuticals, pharmaceuticals, vitamins, genes for modifying amino acid content, herbicide resistance, cold, drought, and heat tolerance, industrial products, oils, protein, carbohydrates, antioxidants, male sterile plants, flowers, fuels, other output traits, genes encoding therapeutically desirable polypeptides or products

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that may be used to treat a condition, a disease, a disorder, a dysfunction, a genetic defect, such as monoclonal antibodies, enzymes, proteases, cytokines, interferons, insulin, erthropoietin, clotting factors, other blood factors or components, viral vectors for gene therapy, virus for vaccines, targets for drug discovery, functional genomics, and proteomics  
 5 analyses and applications, and the like.

Acceptable ligands are any that modulate expression of the gene when binding of the DNA binding domain of the gene expression system according to the invention to the response element in the presence of the ligand results in activation or suppression of expression of the genes. Preferred ligands include an ecdysteroid, such as ecdysone, 20-  
 10 hydroxyecdysone, ponasterone A, muristerone A, and the like, 9-cis-retinoic acid, synthetic analogs of retinoic acid, N,N'-diacylhydrazines such as those disclosed in U. S. Patents No. 6,013,836; 5,117,057; 5,530,028; and 5,378,726; dibenzoylalkyl cyanohydrazines such as those disclosed in European Application No. 461,809; N-alkyl-N,N'-diaroylhydrazines such as those disclosed in U. S. Patent No. 5,225,443; N-acyl-N-alkylcarbonylhydrazines such as  
 15 those disclosed in European Application No. 234,994; N-aroyle-N-alkyl-N'-aroylhydrazines such as those described in U. S. Patent No. 4,985,461; each of which is incorporated herein by reference and other similar materials including 3,5-di-tert-butyl-4-hydroxy-N-isobutylbenzamide, 8-O-acetylharpagide, oxysterols, 22(R) hydroxycholesterol, 24(S) hydroxycholesterol, 25-epoxycholesterol, T0901317, 5-alpha-6-alpha-epoxycholesterol-3-  
 20 sulfate (ECHS), 7-ketocholesterol-3-sulfate, farnesol, bile acids, 1,1-biphosphonate esters, Juvenile hormone III, and the like.

In a preferred embodiment, the ligand for use in Applicants' method of modulating expression of gene is a compound of the formula:



25 wherein:

E is a (C<sub>4</sub>-C<sub>6</sub>)alkyl containing a tertiary carbon or a cyano(C<sub>3</sub>-C<sub>5</sub>)alkyl containing a tertiary carbon;

R<sup>1</sup> is H, Me, Et, i-Pr, F, formyl, CF<sub>3</sub>, CHF<sub>2</sub>, CHCl<sub>2</sub>, CH<sub>2</sub>F, CH<sub>2</sub>Cl, CH<sub>2</sub>OH, CH<sub>2</sub>OMe, CH<sub>2</sub>CN, CN, C<sup>o</sup>CH, 1-propynyl, 2-propynyl, vinyl, OH, OMe, OEt, cyclopropyl,

CF<sub>2</sub>CF<sub>3</sub>, CH=CHCN, allyl, azido, SCN, or SCHF<sub>2</sub>;

R<sup>2</sup> is H, Me, Et, n-Pr, i-Pr, formyl, CF<sub>3</sub>, CHF<sub>2</sub>, CHCl<sub>2</sub>, CH<sub>2</sub>F, CH<sub>2</sub>Cl, CH<sub>2</sub>OH, CH<sub>2</sub>OMe, CH<sub>2</sub>CN, CN, C<sup>o</sup>CH, 1-propynyl, 2-propynyl, vinyl, Ac, F, Cl, OH, OMe, OEt, O-n-Pr, OAc, NMe<sub>2</sub>, NEt<sub>2</sub>, SMe, SEt, SOCF<sub>3</sub>, OCF<sub>2</sub>CF<sub>2</sub>H, COEt, cyclopropyl, CF<sub>2</sub>CF<sub>3</sub>,  
 5 CH=CHCN, allyl, azido, OCF<sub>3</sub>, OCHF<sub>2</sub>, O-i-Pr, SCN, SCHF<sub>2</sub>, SMe, NH-CN, or joined with R<sup>3</sup> and the phenyl carbons to which R<sup>2</sup> and R<sup>3</sup> are attached to form an ethylenedioxy, a dihydrofuryl ring with the oxygen adjacent to a phenyl carbon, or a dihydropyryl ring with the oxygen adjacent to a phenyl carbon;

10 R<sup>3</sup> is H, Et, or joined with R<sup>2</sup> and the phenyl carbons to which R<sup>2</sup> and R<sup>3</sup> are attached to form an ethylenedioxy, a dihydrofuryl ring with the oxygen adjacent to a phenyl carbon, or a dihydropyryl ring with the oxygen adjacent to a phenyl carbon;

R<sup>4</sup>, R<sup>5</sup>, and R<sup>6</sup> are independently H, Me, Et, F, Cl, Br, formyl, CF<sub>3</sub>, CHF<sub>2</sub>, CHCl<sub>2</sub>, CH<sub>2</sub>F, CH<sub>2</sub>Cl, CH<sub>2</sub>OH, CN, C<sup>o</sup>CH, 1-propynyl, 2-propynyl, vinyl, OMe, OEt, SMe, or SEt.

In another preferred embodiment, the ligand for use in Applicants' method of  
 15 modulating expression of gene is an ecdysone, 20-hydroxyecdysone, ponasterone A, muristerone A, an oxysterol, a 22(R) hydroxycholesterol, 24(S) hydroxycholesterol, 25-epoxycholesterol, T0901317, 5-alpha-6-alpha-epoxycholesterol-3-sulfate (ECHS), 7-ketocholesterol-3-sulfate, farnesol, bile acids, 1,1-biphosphonate esters, or Juvenile hormone III.

20 In another preferred embodiment, a second ligand may be used in addition to the first ligand discussed above in Applicants' method of modulating expression of a gene. Preferably, this second ligand is 9-cis-retinoic acid or a synthetic analog of retinoic acid.

#### HOST CELLS AND NON-HUMAN ORGANISMS OF THE INVENTION

25 As described above, the gene expression modulation system of the present invention may be used to modulate gene expression in a host cell. Expression in transgenic host cells may be useful for the expression of various genes of interest. Applicants' invention provides for modulation of gene expression in prokaryotic and eukaryotic host cells. Expression in transgenic host cells is useful for the expression of various polypeptides of interest including  
 30 but not limited to antigens produced in plants as vaccines, enzymes like alpha-amylase, phytase, glucanase, and xylanase, genes for resistance against insects, nematodes, fungi, bacteria, viruses, and abiotic stresses, antigens, nutraceuticals, pharmaceuticals, vitamins, genes for modifying amino acid content, herbicide resistance, cold, drought, and heat tolerance, industrial products, oils, protein, carbohydrates, antioxidants, male sterile plants,

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flowers, fuels, other output traits, therapeutic polypeptides, pathway intermediates; for the modulation of pathways already existing in the host for the synthesis of new products heretofore not possible using the host; cell based assays; functional genomics assays, biotherapeutic protein production, proteomics assays, and the like. Additionally the gene products may be useful for conferring higher growth yields of the host or for enabling an alternative growth mode to be utilized.

Thus, Applicants' invention provides an isolated host cell comprising a gene expression system according to the invention. The present invention also provides an isolated host cell comprising a gene expression cassette according to the invention. Applicants' invention also provides an isolated host cell comprising a polynucleotide or a polypeptide according to the invention. The present invention also relates to a host cell transfected with an expression vector according to the invention. The host cell may be a bacterial cell, a fungal cell, a nematode cell, an insect cell, a fish cell, a plant cell, an avian cell, an animal cell, or a mammalian cell. In still another embodiment, the invention relates to a method for producing a nuclear receptor ligand binding domain comprising a substitution mutation, wherein the method comprises culturing the host cell as described above in culture medium under conditions permitting expression of a polynucleotide encoding the nuclear receptor ligand binding domain comprising a substitution mutation, and isolating the nuclear receptor ligand binding domain comprising a substitution mutation from the culture.

In a specific embodiment, the isolated host cell is a prokaryotic host cell or a eukaryotic host cell. In another specific embodiment, the isolated host cell is an invertebrate host cell or a vertebrate host cell. Preferably, the host cell is selected from the group consisting of a bacterial cell, a fungal cell, a yeast cell, a nematode cell, an insect cell, a fish cell, a plant cell, an avian cell, an animal cell, and a mammalian cell. More preferably, the host cell is a yeast cell, a nematode cell, an insect cell, a plant cell, a zebrafish cell, a chicken cell, a hamster cell, a mouse cell, a rat cell, a rabbit cell, a cat cell, a dog cell, a bovine cell, a goat cell, a cow cell, a pig cell, a horse cell, a sheep cell, a simian cell, a monkey cell, a chimpanzee cell, or a human cell. Examples of preferred host cells include, but are not limited to, fungal or yeast species such as *Aspergillus*, *Trichoderma*, *Saccharomyces*, *Pichia*, *Candida*, *Hansenula*, or bacterial species such as those in the genera *Synechocystis*, *Synechococcus*, *Salmonella*, *Bacillus*, *Acinetobacter*, *Rhodococcus*, *Streptomyces*, *Escherichia*, *Pseudomonas*, *Methylobacter*, *Methylobacter*, *Alcaligenes*, *Synechocystis*, *Anabaena*, *Thiobacillus*, *Methanobacterium* and *Klebsiella*; plant species selected from the group consisting of an apple, *Arabidopsis*, bajra, banana, barley, beans, beet, blackgram,

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chickpea, chili, cucumber, eggplant, favabean, maize, melon, millet, mungbean, oat, okra, *Panicum*, papaya, peanut, pea, pepper, pigeonpea, pineapple, *Phaseolus*, potato, pumpkin, rice, sorghum, soybean, squash, sugarcane, sugarbeet, sunflower, sweet potato, tea, tomato, tobacco, watermelon, and wheat; animal; and mammalian host cells.

5 In a specific embodiment, the host cell is a yeast cell selected from the group consisting of a *Saccharomyces*, a *Pichia*, and a *Candida* host cell.

In another specific embodiment, the host cell is a *Caenorhabdus elegans* nematode cell.

In another specific embodiment, the host cell is an insect cell.

10 In another specific embodiment, the host cell is a plant cell selected from the group consisting of an apple, *Arabidopsis*, bajra, banana, barley, beans, beet, blackgram, chickpea, chili, cucumber, eggplant, favabean, maize, melon, millet, mungbean, oat, okra, *Panicum*, papaya, peanut, pea, pepper, pigeonpea, pineapple, *Phaseolus*, potato, pumpkin, rice, sorghum, soybean, squash, sugarcane, sugarbeet, sunflower, sweet potato, tea, tomato, tobacco, watermelon, and wheat cell.

In another specific embodiment, the host cell is a zebrafish cell.

In another specific embodiment, the host cell is a chicken cell.

In another specific embodiment, the host cell is a mammalian cell selected from the group consisting of a hamster cell, a mouse cell, a rat cell, a rabbit cell, a cat cell, a dog cell, 20 a bovine cell, a goat cell, a cow cell, a pig cell, a horse cell, a sheep cell, a monkey cell, a chimpanzee cell, and a human cell.

Host cell transformation is well known in the art and may be achieved by a variety of methods including but not limited to electroporation, viral infection, plasmid/vector transfection, non-viral vector mediated transfection, *Agrobacterium*-mediated transformation, 25 particle bombardment, and the like. Expression of desired gene products involves culturing the transformed host cells under suitable conditions and inducing expression of the transformed gene. Culture conditions and gene expression protocols in prokaryotic and eukaryotic cells are well known in the art (see General Methods section of Examples). Cells may be harvested and the gene products isolated according to protocols specific for the gene 30 product.

In addition, a host cell may be chosen which modulates the expression of the inserted polynucleotide, or modifies and processes the polypeptide product in the specific fashion desired. Different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, cleavage [*e.g.*, of

signal sequence]) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce a non-glycosylated core protein product. However, a polypeptide expressed in bacteria may not be properly folded.

- 5 Expression in yeast can produce a glycosylated product. Expression in eukaryotic cells can increase the likelihood of "native" glycosylation and folding of a heterologous protein. Moreover, expression in mammalian cells can provide a tool for reconstituting, or constituting, the polypeptide's activity. Furthermore, different vector/host expression systems may affect processing reactions, such as proteolytic cleavages, to a different extent.

- 10 Applicants' invention also relates to a non-human organism comprising an isolated host cell according to the invention. In a specific embodiment, the non-human organism is a prokaryotic organism or a eukaryotic organism. In another specific embodiment, the non-human organism is an invertebrate organism or a vertebrate organism.

- 15 Preferably, the non-human organism is selected from the group consisting of a bacterium, a fungus, a yeast, a nematode, an insect, a fish, a plant, a bird, an animal, and a mammal. More preferably, the non-human organism is a yeast, a nematode, an insect, a plant, a zebrafish, a chicken, a hamster, a mouse, a rat, a rabbit, a cat, a dog, a bovine, a goat, a cow, a pig, a horse, a sheep, a simian, a monkey, or a chimpanzee.

- 20 In a specific embodiment, the non-human organism is a yeast selected from the group consisting of *Saccharomyces*, *Pichia*, and *Candida*.

In another specific embodiment, the non-human organism is a *Caenorhabditis elegans* nematode.

- 25 In another specific embodiment, the non-human organism is a plant selected from the group consisting of an apple, *Arabidopsis*, bajra, banana, barley, beans, beet, blackgram, chickpea, chili, cucumber, eggplant, favabean, maize, melon, millet, mungbean, oat, okra, *Panicum*, papaya, peanut, pea, pepper, pigeonpea, pineapple, *Phaseolus*, potato, pumpkin, rice, sorghum, soybean, squash, sugarcane, sugarbeet, sunflower, sweet potato, tea, tomato, tobacco, watermelon, and wheat.

- 30 In another specific embodiment, the non-human organism is a *Mus musculus* mouse.

#### MEASURING GENE EXPRESSION/TRANSCRIPTION

One useful measurement of Applicants' methods of the invention is that of the transcriptional state of the cell including the identities and abundances of RNA, preferably mRNA species. Such measurements are conveniently conducted by measuring cDNA

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abundances by any of several existing gene expression technologies.

Nucleic acid array technology is a useful technique for determining differential mRNA expression. Such technology includes, for example, oligonucleotide chips and DNA microarrays. These techniques rely on DNA fragments or oligonucleotides which correspond to different genes or cDNAs which are immobilized on a solid support and hybridized to probes prepared from total mRNA pools extracted from cells, tissues, or whole organisms and converted to cDNA. Oligonucleotide chips are arrays of oligonucleotides synthesized on a substrate using photolithographic techniques. Chips have been produced which can analyze for up to 1700 genes. DNA microarrays are arrays of DNA samples, typically PCR products, that are robotically printed onto a microscope slide. Each gene is analyzed by a full or partial-length target DNA sequence. Microarrays with up to 10,000 genes are now routinely prepared commercially. The primary difference between these two techniques is that oligonucleotide chips typically utilize 25-mer oligonucleotides which allow fractionation of short DNA molecules whereas the larger DNA targets of microarrays, approximately 1000 base pairs, may provide more sensitivity in fractionating complex DNA mixtures.

Another useful measurement of Applicants' methods of the invention is that of determining the translation state of the cell by measuring the abundances of the constituent protein species present in the cell using processes well known in the art.

Where identification of genes associated with various physiological functions is desired, an assay may be employed in which changes in such functions as cell growth, apoptosis, senescence, differentiation, adhesion, binding to a specific molecules, binding to another cell, cellular organization, organogenesis, intracellular transport, transport facilitation, energy conversion, metabolism, myogenesis, neurogenesis, and/or hematopoiesis is measured.

In addition, selectable marker or reporter gene expression may be used to measure gene expression modulation using Applicants' invention.

Other methods to detect the products of gene expression are well known in the art and include Southern blots (DNA detection), dot or slot blots (DNA, RNA), northern blots (RNA), RT-PCR (RNA), western blots (polypeptide detection), and ELISA (polypeptide) analyses. Although less preferred, labeled proteins can be used to detect a particular nucleic acid sequence to which it hybridizes.

In some cases it is necessary to amplify the amount of a nucleic acid sequence. This may be carried out using one or more of a number of suitable methods including, for example, polymerase chain reaction ("PCR"), ligase chain reaction ("LCR"), strand

displacement amplification ("SDA"), transcription-based amplification, and the like. PCR is carried out in accordance with known techniques in which, for example, a nucleic acid sample is treated in the presence of a heat stable DNA polymerase, under hybridizing conditions, with one pair of oligonucleotide primers, with one primer hybridizing to one

5 strand (template) of the specific sequence to be detected. The primers are sufficiently complementary to each template strand of the specific sequence to hybridize therewith. An extension product of each primer is synthesized and is complementary to the nucleic acid template strand to which it hybridized. The extension product synthesized from each primer can also serve as a template for further synthesis of extension products using the same

10 primers. Following a sufficient number of rounds of synthesis of extension products, the sample may be analyzed as described above to assess whether the sequence or sequences to be detected are present.

The present invention may be better understood by reference to the following non-

15 limiting Examples, which are provided as exemplary of the invention.

## EXAMPLES

### GENERAL METHODS

20 Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, (1989) (Maniatis) and by T. J. Silhavy, M. L. Bannan, and L. W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1984) and by

25 Ausubel, F. M. et al., *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley-Interscience (1987).

Methods for plant tissue culture, transformation, plant molecular biology, and plant, general molecular biology may be found in *Plant Tissue Culture Concepts and Laboratory Exercises* edited by RN Trigiano and DJ Gray, 2<sup>nd</sup> edition, 2000, CRC press, New York;

30 *Agrobacterium Protocols* edited by KMA Gartland and MR Davey, 1995, Humana Press, Totowa, New Jersey; *Methods in Plant Molecular Biology*, P. Maliga et al., 1995, Cold Spring Harbor Lab Press, New York; and *Molecular Cloning*, J. Sambrook et al., 1989, Cold Spring Harbor Lab Press, New York.

Materials and methods suitable for the maintenance and growth of bacterial cultures

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are well known in the art. Techniques suitable for use in the following examples may be found as set out in *Manual of Methods for General Bacteriology* (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994)) or by Thomas D. Brock in *Biotechnology: A Textbook of Industrial Microbiology*, Second Edition, Sinauer Associates, Inc., Sunderland, MA (1989). All reagents, restriction enzymes and materials used for the growth and maintenance of host cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

- 10 Manipulations of genetic sequences may be accomplished using the suite of programs available from the Genetics Computer Group Inc. (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI). Where the GCG program "Pileup" is used the gap creation default value of 12, and the gap extension default value of 4 may be used. Where the CGC "Gap" or "Bestfit" programs is used the default gap creation penalty of 50
- 15 and the default gap extension penalty of 3 may be used. In any case where GCG program parameters are not prompted for, in these or any other GCG program, default values may be used.

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "μl" means microliter(s), "ml" means milliliter(s), "L" means liter(s), "μM" means micromolar, "mM" means millimolar, "μg"

20 means microgram(s), "mg" means milligram(s), "A" means adenine or adenosine, "T" means thymine or thymidine, "G" means guanine or guanosine, "C" means cytidine or cytosine, "x g" means times gravity, "nt" means nucleotide(s), "aa" means amino acid(s), "bp" means base pair(s), "kb" means kilobase(s), "k" means kilo, "μ" means micro, and "°C" means

25 degrees Celsius.

### EXAMPLE 1

Applicants' improved EcR-based inducible gene modulation system was developed

30 for use in various applications including gene therapy, expression of proteins of interest in host cells, production of transgenic organisms, and cell-based assays. This Example describes the construction and evaluation of several gene expression cassettes for use in the EcR-based inducible gene expression system of the invention.

In various cellular backgrounds, including mammalian cells, insect ecdysone receptor

(EcR) heterodimerizes with retinoid X receptor (RXR) and, upon binding of ligand, transactivates genes under the control of ecdysone response elements. Applicants constructed several EcR-based gene expression cassettes based on the spruce budworm *Choristoneura fumiferana* EcR ("CfEcR"; full length polynucleotide and amino acid sequences are set forth in SEQ ID NO: 58 and SEQ ID NO: 59, respectively), *C. fumiferana* ultraspiracle ("CfUSP"; full length polynucleotide and amino acid sequences are set forth in SEQ ID NO: 60 and SEQ ID NO: 61, respectively), and mouse *Mus musculus* RXR $\alpha$  (MmRXR $\alpha$ ; full length polynucleotide and amino acid sequences are set forth in SEQ ID NO: 62 and SEQ ID NO: 63, respectively). The prepared receptor constructs comprise a ligand binding domain of EcR and of RXR or of USP; a DNA binding domain of GAL4 or of EcR; and an activation domain of VP16. The reporter constructs include a reporter gene, luciferase or LacZ, operably linked to a synthetic promoter construct that comprises either GAL4 or EcR/USP binding sites (response elements). Various combinations of these receptor and reporter constructs were cotransfected into CHO, NIH3T3, CV1 and 293 cells. Gene induction potential (magnitude of induction) and ligand specificity and sensitivity were examined using four different ligands: two steroidal ligands (ponasterone A and muristerone A) and two non-steroidal ligands (N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine and N-(3,4-(1,2-ethylenedioxy)-2-methylbenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine) in a dose-dependent induction of reporter gene expression in the transfected cells. Reporter gene expression activities were assayed at 24 hours or 48 hours after ligand addition.

**Gene Expression Cassettes:** Ecdysone receptor-based, chemically inducible gene expression cassettes (switches) were constructed as followed, using standard cloning methods available in the art. The following is brief description of preparation and composition of each switch.

1.1 - GAL4EcR/VP16RXR: The D, E, and F domains from spruce budworm *Choristoneura fumiferana* EcR ("CfEcRDEF"; SEQ ID NO: 3) were fused to GAL4 DNA binding domain ("DNABD"; SEQ ID NO: 43) and placed under the control of an SV40e promoter (SEQ ID NO: 64). The DEF domains from mouse (*Mus musculus*) RXR ("MmRXRDEF"; SEQ ID NO: 22) were fused to the activation domain from VP16 ("VP16AD"; SEQ ID NO: 49) and placed under the control of an SV40e promoter (SEQ ID NO: 64). Five consensus GAL4 binding sites ("5XGAL4RE"; comprising 5, GAL4RE comprising SEQ ID NO: 56) were fused to a synthetic E1b minimal promoter (SEQ ID NO: 65) and placed upstream of the luciferase gene (SEQ ID NO: 66).

1.2 - GAL4EcR/VP16USP: This construct was prepared in the same way as in switch 1.1 above except MmRXRDEF was replaced with the D, E and F domains from spruce budworm USP ("CfUSPDEF"; SEQ ID NO: 67). The constructs used in this example are similar to those disclosed in U. S. Patent No. 5,880,333 except that *Choristoneura fumiferana* USP  
 5 rather than *Drosophila melanogaster* USP was utilized.

1.3 - GAL4RXR/VP16CfEcR: MmRXRDEF (SEQ ID NO: 22) was fused to a GAL4DNABD (SEQ ID NO: 43) and CfEcRCDEF (SEQ ID NO: 1) was fused to a VP16AD (SEQ ID NO: 49).

1.4 - GAL4RXR/VP16DmEcR: This construct was prepared in the same way as switch 1.3  
 10 except CfEcRCDEF was replaced with DmEcRCDEF (SEQ ID NO: 6).

1.5 - GAL4USP/VP16CfEcR: This construct was prepared in the same way as switch 1.3 except MmRXRDEF was replaced with CfUSPDEF (SEQ ID NO: 67).

1.6 - GAL4RXRCfEcRVP16: This construct was prepared so that both the GAL4 DNABD and the VP16AD were placed on the same molecule. GAL4DNABD (SEQ ID NO: 43) and  
 15 VP16AD (SEQ ID NO: 49) were fused to CfEcRDEF (SEQ ID NO: 3) at N-and C-termini respectively. The fusion was placed under the control of an SV40e promoter (SEQ ID NO: 64).

1.7 - VP16CfEcR: This construct was prepared such that CfEcRCDEF (SEQ ID NO: 1) was fused to VP16AD (SEQ ID NO: 49) and placed under the control of an SV40e promoter  
 20 (SEQ ID NO: 64). Six ecdysone response elements ("EcRE"; SEQ ID NO: 55) from the hsp27 gene were placed upstream of the promoter and a luciferase gene (SEQ ID NO: 66). This switch most probably uses endogenous RXR.

1.8 - DmVgRXR: This system was purchased from Invitrogen Corp., Carlsbad, California. It comprises a *Drosophila melanogaster* EcR ("DmEcR") with a modified DNABD fused to  
 25 VP16AD and placed under the control of a CMV promoter (SEQ ID NO: 68). Full length MmRXR (SEQ ID NO: 62) was placed under the control of the RSV promoter (SEQ ID NO: 69). The reporter, pIND(SP1)LacZ, contains five copies of a modified ecdysone response element ("EcRE", E/GRE), three copies of an SP1 enhancer, and a minimal heat shock promoter, all of which were placed upstream to the LacZ reporter gene.

30 1.9 - CfVgRXR: This example was prepared in the same way as switch 1.8 except DmEcR was replaced with a truncated CfEcR comprising a partial A/B domain and the complete CDEF domains [SEQ ID NO: 70 (polynucleotide) and SEQ ID NO: 71 (polypeptide)].

1.10 - CfVgRXRdel: This example was prepared in the same way as switch 1.9 except MmRXR (SEQ ID NO: 62) was deleted.

**Cell lines:** Four cell lines: CHO, Chinese hamster *Cricetulus griseus* ovarian cell line; NIH3T3 (3T3) mouse *Mus musculus* cell line; 293 human *Homo sapiens* kidney cell line, and CV1 African green monkey kidney cell line were used in these experiments. Cells were maintained in their respective media and were subcultured when they reached 60% confluency. Standard methods for culture and maintenance of the cells were followed.

**Transfections:** Several commercially available lipofactors as well as electroporation methods were evaluated and the best conditions for transfection of each cell line were developed. CHO, NIH3T3, 293 and CV1 cells were grown to 60% confluency. DNAs corresponding to the various switch constructs outlined in Examples 1.1 through 1.10 were transfected into CHO cells, NIH3T3 cells, 293 cells, or CV1 cells as follows.

**CHO cells:** Cells were harvested when they reach 60-80% confluency and plated in 6- or 12- or 24- well plates at 250,000, 100,000, or 50,000 cells in 2.5, 1.0, or 0.5 ml of growth medium containing 10% Fetal bovine serum respectively. The next day, the cells were rinsed with growth medium and transfected for four hours. LipofectAMINE™ 2000 (Life Technologies Inc.) was found to be the best transfection reagent for these cells. For 12- well plates, 4 µl of LipofectAMINE™ 2000 was mixed with 100 µl of growth medium. 1.0 µg of reporter construct and 0.25 µg of receptor construct(s) were added to the transfection mix. A second reporter construct was added [pTKRL (Promega), 0.1 µg/transfection mix] and comprised a *Renilla* luciferase gene (SEQ ID NO: 72) operably linked and placed under the control of a thymidine kinase (TK) constitutive promoter and was used for normalization. The contents of the transfection mix were mixed in a vortex mixer and let stand at room temperature for 30 min. At the end of incubation, the transfection mix was added to the cells maintained in 400 µl growth medium. The cells were maintained at 37°C and 5% CO<sub>2</sub> for four hours. At the end of incubation, 500 µl of growth medium containing 20% FBS and either DMSO (control) or a DMSO solution of appropriate ligands were added and the cells were maintained at 37 °C and 5% CO<sub>2</sub> for 24-48 hr. The cells were harvested and reporter activity was assayed. The same procedure was followed for 6 and 24 well plates as well except all the reagents were doubled for 6 well plates and reduced to half for 24-well plates.

**NIH3T3 Cells:** Superfect™ (Qiagen Inc.) was found to be the best transfection reagent for 3T3 cells. The same procedures described for CHO cells were followed for 3T3 cells as well with two modifications. The cells were plated when they reached 50% confluency. 125,000 or 50,000 or 25,000 cells were plated per well of 6- or 12- or 24-well plates respectively. The

GAI4EcR/VP16RXR and reporter vector DNAs were transfected into NIH3T3 cells, the transfected cells were grown in medium containing PonA, MurA, N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-t-butylhydrazine, or N-(3,4-(1,2-ethylenedioxy)-2-methylbenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine for 48 hr.

- 5 The ligand treatments were performed as described in the CHO cell section above.

293 Cells: LipofectAMINE™ 2000 (Life Technologies) was found to be the best lipofactor for 293 cells. The same procedures described for CHO were followed for 293 cells except that the cells were plated in biocoated plates to avoid clumping. The ligand treatments were performed as described in the CHO cell section above.

- 10 CV1 Cells: LipofectAMINE™ plus (Life Technologies) was found to be the best lipofactor for CV1 cells. The same procedures described for NIH3T3 cells were followed for CV1 cells

**Ligands:** Ponasterone A and Muristerone A were purchased from Invitrogen and Sigma Chemical Company. The two non-steroids N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-

- 15 dimethylbenzoyl)-N'-t-butylhydrazine, or N-(3,4-(1,2-ethylenedioxy)-2-methylbenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine are synthetic stable ecdysteroids synthesized at Rohm and Haas Company. All ligands were dissolved in DMSO and the final concentration of DMSO was maintained at 0.1% in both controls and treatments.

- 20 **Reporter Assays:** Cells were harvested 24-48 hr after adding ligands. 125, 250, or 500 µl of passive lysis buffer (part of Dual-luciferase™ reporter assay system from Promega Corporation) were added to each well of 24- or 12- or 24-well plates respectively. The plates were placed on a rotary shaker for 15 min. Twenty µl of lysate was assayed. Luciferase activity was measured using Dual-luciferase™ reporter assay system from Promega
- 25 Corporation following the manufacturer's instructions. β-Galactosidase was measured using Galacto-Star™ assay kit from TROPIX following the manufacturer's instructions. All luciferase and β-galactosidase activities were normalized using *Renilla* luciferase as a standard. Fold activities were calculated by dividing normalized relative light units ("RLU") in ligand treated cells with normalized RLU in DMSO treated cells (untreated control).

- 30 The results of these experiments are provided in the following tables.

**Table 1**

**Transactivation of reporter genes through various switches in CHO cells**

Composition of Switch	Mean Fold Activation with 50 $\mu$ M N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-t-butylhydrazine
1.1 GAL4EcR + VP16RXR pGAL4RELuc	267
1.2 GAL4EcR + VP16USP pGAL4RELuc	2
1.3 GAL4RXR + VP16CfEcR pGAL4RELuc	85
1.4 GAL4RXR + VP16DmEcR pGAL4RELuc	312
1.5 GAL4USP + VP16CfEcR pGAL4RELuc	2
1.6 GAL4CfEcRVP16 pGAL4RELuc	9
1.7 VP16CfEcR pEcRELuc	36
1.8 DmVgRXR + MmRXR pIND(SP1)LacZ	14
1.9 CfVgRXR + MmRXR pIND(SP1)LacZ	27
1.10 CfVgRXR pIND(SP1)LacZ	29

Table 2

## Transactivation of reporter genes through various switches in 3T3 cells

Composition of Switch	Mean Fold Activation Through N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-t-butylhydrazine
1.1 GAL4EcR + VP16RXR pGAL4RELuc	1118
1.2 GAL4EcR + VP16USP pGAL4RELuc	2
1.3 GAL4RXR + VP16CfEcR pGAL4RELuc	47
1.4 GAL4RXR + VP16DmEcR pGAL4RELuc	269
1.5 GAL4USP + VP16CfEcR pGAL4RELuc	3
1.6 GAL4CfEcRVP16 pGAL4RELuc	7
1.7 VP16CfEcR pEcRELuc	1
1.8 DmVgRXR + MmRXR pIND(SP1)LacZ	21
1.9 CfVgRXR + MmRXR pIND(SP1)LacZ	19
1.10 CfVgRXR pIND(SP1)LacZ	2

Table 3

Transactivation of reporter genes through various switches in 293 cells

Composition of Switch	Mean Fold Activation Through N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-t-butylhydrazine
1.1 GAL4EcR + VP16RXR pGAL4RELuc	125
1.2 GAL4EcR + VP16USP pGAL4RELuc	2
1.3 GAL4RXR + VP16CfEcR pGAL4RELuc	17
1.4 GAL4RXR + VP16DmEcR pGAL4RELuc	3
1.5 GAL4USP + VP16CfEcR pGAL4RELuc	2
1.6 GAL4CfEcRVP16 pGAL4RELuc	3
1.7 VP16CfEcR pEcRELuc	2
1.8 DmVgRXR + MmRXR pIND(SP1)LacZ	21
1.9 CfVgRXR + MmRXR pIND(SP1)LacZ	12
1.10 CfVgRXR pIND(SP1)LacZ	3

Table 4

5 Transactivation of reporter genes through various switches in CV1 cells

Composition of Switch	Mean Fold Activation Through N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-t-butylhydrazine
1.1 GAL4EcR + VP16RXR pGAL4RELuc	279
1.2 GAL4EcR + VP16USP pGAL4RELuc	2
1.3 GAL4RXR + VP16CfEcR pGAL4RELuc	25
1.4 GAL4RXR + VP16DmEcR pGAL4RELuc	80
1.5 GAL4USP + VP16CfEcR pGAL4RELuc	3
1.6 GAL4CfEcRVP16 pGAL4RELuc	6
1.7 VP16CfEcR pEcRELuc	1
1.8 DmVgRXR + MmRXR pIND(SP1)LacZ	12
1.9 CfVgRXR + MmRXR pIND(SP1)LacZ	7
1.10 CfVgRXR	1

pIND(SP1)LacZ

**Table 5**  
**Transactivation of reporter gene GAL4CfEcRDEF/VP16MmRXRDEF (switch 1.1)**  
**through steroids and non-steroids in 3T3 cells.**

Ligand	Mean Fold Induction at 1.0 $\mu$ M Concentration
1. Ponasterone A	1.0
2. Muristerone A	1.0
3. N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine	116
4. N'-(3,4-(1,2-ethylenedioxy)-2-methylbenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine	601

**Table 6**  
**Transactivation of reporter gene GAL4MmRXRDEF/VP16CfEcRCDEF (switch 1.3)**  
**through steroids and non-steroids in 3T3 cells.**

Ligand	Mean Fold Induction at 1.0 $\mu$ M Concentration
1. Ponasterone A	1.0
2. Muristerone A	1.0
3. N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine	71
4. N'-(3,4-(1,2-ethylenedioxy)-2-methylbenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine	54

Applicants' results demonstrate that the non-steroidal ecdysone agonists, N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine and N'-(3,4-(1,2-ethylenedioxy)-2-methylbenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine, were more potent activators of CfEcR as compared to *Drosophila melanogaster* EcR (DmEcR). (see Tables 1-4). Also, in the mammalian cell lines tested, MmRXR performed better than CfUSP as a heterodimeric partner for CfEcR. (see Tables 1-4). Additionally, Applicants' inducible gene expression modulation system performed better when exogenous MmRXR was used than when the system relied only on endogenous RXR levels (see Tables 1-4).

Applicants' results also show that in a CfEcR-based inducible gene expression system, the non-steroidal ecdysone agonists induced reporter gene expression at a lower concentration (i.e., increased ligand sensitivity) as compared to the steroid ligands, ponasterone A and muristerone A (see Tables 5 and 6).

Out of 10 EcR based gene switches tested, the GAL4EcR/VP16RXR switch (Switch



1.1) performed better than any other switch in all four cell lines examined and was more sensitive to non-steroids than steroids. The results also demonstrate that placing the activation domain (AD) and DNA binding domain (DNABD) on each of the two partners reduced background when compared to placing both AD and DNABD together on one of the two partners. Therefore, a switch format where the AD and DNABD are separated between two partners, works well for EcR-based gene switch applications.

In addition, the MmRXR/EcR-based switches performed better than CfUSP/EcR-based switches, which have a higher background activity than the MmRXR/EcR switches in the absence of ligand.

Finally, the GAL4EcR/VP16RXR switch (Switch 1.1) was more sensitive to non-steroid ligands than to the steroid ligands (see Tables 5 and 6). In particular, steroid ligands initiated transactivation at concentrations of 50  $\mu$ M, whereas the non-steroid ligands initiated transactivation at less than 1  $\mu$ M (submicromolar) concentration.

## EXAMPLE 2

This Example describes Applicants' further analysis of truncated EcR and RXR polypeptides in the improved EcR-based inducible gene expression system of the invention. To identify the best combination and length of two receptors that give a switch with a) maximum induction in the presence of ligand; b) minimum background in the absence of ligand; c) highly sensitive to ligand concentration; and d) minimum cross-talk among ligands and receptors, Applicants made and analyzed several truncation mutations of the CfEcR and MmRXR receptor polypeptides in NIH3T3 cells.

Briefly, polynucleotides encoding EcR or RXR receptors were truncated at the junctions of A/B, C, D, E and F domains and fused to either a GAL4 DNA binding domain encoding polynucleotide (SEQ ID NO: 43) for CfEcR, or a VP16 activation domain encoding polynucleotide (SEQ ID NO: 49) for MmRXR as described in Example 1. The resulting receptor truncation/fusion polypeptides were assayed in NIH3T3 cells. Plasmid pFRLUC (Stratagene) encoding a luciferase polypeptide was used as a reporter gene construct and pTKRL (Promega) encoding a *Renilla* luciferase polypeptide under the control of the constitutive TK promoter was used to normalize the transfections as described above. The analysis was performed in triplicates and mean luciferase counts were determined as described above.

### Gene Expression Cassettes Encoding Truncated Ecdysone Receptor Polypeptides

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Gene expression cassettes comprising polynucleotides encoding either full length or truncated CfEcR polypeptides fused to a GAL4 DNA binding domain (SEQ ID NO: 43): GAL4CfEcRA/BCDEF (full length CfEcRA/BCDEF; SEQ ID NO: 58), GAL4CfEcRCDEF (CfEcRCDEF; SEQ ID NO: 1), GAL4CfEcR1/2CDEF (CfEcR1/2CDEF; SEQ ID NO: 2),

5 GAL4CfEcRDEF (CfEcRDEF; SEQ ID NO: 3), GAL4CfEcREF (CfEcREF; SEQ ID NO: 4), and GAL4CfEcRDE (CfEcRDE; SEQ ID NO: 5) were transfected into NIH3T3 cells along with VP16MmRXRDEF (constructed as in Example 1.1; Figure 11) or VP16MmRXREF [constructed as in Example 1.1 except that MmRXRDEF was replaced with MmRXREF (SEQ ID NO: 23); Figure 12], and pFRLuc and pTKRL plasmid DNAs. The transfected

10 cells were grown in the presence 0, 1, 5 or 25 uM of N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine or PonA for 48 hr. The cells were harvested, lysed and luciferase reporter activity was measured in the cell lysates. Total fly luciferase relative light units are presented. The number on the top of each bar is the maximum fold induction for that treatment.

15 Applicants' results show that the EF domain of MmRXR is sufficient and performs better than DEF domains of this receptor (see Figures 11 and 12). Applicants have also shown that, in general, EcR/RXR receptor combinations are insensitive to PonA (see Figures 11 and 12). As shown in the Figures 11 and 12, the GAL4CfEcRCDEF hybrid polypeptide (SEQ ID NO: 7) performed better than any other CfEcR hybrid polypeptide.

#### 20 Gene Expression Cassettes Encoding Truncated Retinoid X Receptor Polypeptides

Gene expression cassettes comprising polynucleotides encoding either full length or truncated MmRXR polypeptides fused to a VP16 transactivation domain (SEQ ID NO: 49): VP16MmRXRA/BCDEF (full length MmRXRA/BCDEF; SEQ ID NO: 62), VP16MmRXRCDEF (MmRXRCDEF; SEQ ID NO: 21), VP16MmRXRDEF

25 (MmRXRDEF; SEQ ID NO: 22), VP16MmRXREF (MmRXREF; SEQ ID NO: 23), VP16MmRXRBam-EF ("MmRXRBam-EF" or "MmRXR-truncatedEF"; SEQ ID NO: 24), and VP16MmRXRAF2del ("MmRXRAF2del" or "MmRXR-E"; SEQ ID NO: 25) constructs were transfected into NIH3T3 cells along with GAL4CfEcRCDEF (constructed as in Example 1.1; Figure 13) or GAL4CfEcRDEF [constructed as in Example 1.1 except

30 CfEcRCDEF was replaced with CfEcRDEF (SEQ ID NO: 3); Figure 14], pFRLuc and pTKRL plasmid DNAs as described above. The transfected cells were grown in the presence 0, 1, 5 and 25 uM of N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine or PonA for 48 hr. The cells were harvested and lysed and reporter activity was measured in the cell lysate. Total fly luciferase relative light units are presented. The

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number on top of each bar is the maximum fold induction in that treatment.

Of all the truncations of MmRXR tested, Applicants' results show that the MmRXREF receptor was the best partner for CfEcR (Figures 13 and 14). CfEcRCDEF showed better induction than CfEcRDEF using MmRXREF. Deleting AF2 (abbreviated "EF-AF2del") or helices 1-3 of the E domain (abbreviated "EF-Bamdel") resulted in an RXR receptor that reduced gene induction and ligand sensitivity when partnered with either CfEcRCDEF (Figure 13) or CfEcRDEF (Figure 14) in NIH3T3 cells. In general, the CfEcR/RXR-based switch was much more sensitive to the non-steroid N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine than to the steroid PonA.

### EXAMPLE 3

This Example describes Applicants' further analysis of gene expression cassettes encoding truncated EcR or RXR receptor polypeptides that affect either ligand binding activity or ligand sensitivity, or both. Briefly, six different combinations of chimeric receptor pairs, constructed as described in Examples 1 and 2, were further analyzed in a single experiment in NIH3T3 cells. These six receptor pair combinations and their corresponding sample numbers are depicted in Table 7.

**Table 7**

**CfEcR + MmRXR Truncation Receptor Combinations in NIH3T3 Cells**

<b>Figure 15 X-Axis Sample No.</b>	<b>EcR Polypeptide Construct</b>	<b>RXR Polypeptide Construct</b>
Samples 1 and 2	GAL4CfEcRCDEF	VP16RXRA/BCDEF (Full length)
Samples 3 and 4	GAL4CfEcRCDEF	VP16RXRDEF
Samples 5 and 6	GAL4CfEcRCDEF	VP16RXREF
Samples 7 and 8	GAL4CfEcRDEF	VP16RXRA/BCDEF (Full length)
Samples 9 and 10	GAL4CfEcRDEF	VP16RXRDEF
Samples 11 and 12	GAL4CfEcRDEF	VP16RXREF

The above receptor construct pairs, along with the reporter plasmid pFRLuc were transfected into NIH3T3 cells as described above. The six CfEcR truncation receptor combinations were duplicated into two groups and treated with either steroid (odd numbers on x-axis of Figure 15) or non-steroid (even numbers on x-axis of Figure 15). In particular, the cells were grown in media containing 0, 1, 5 or 25 uM PonA (steroid) or N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine (non-steroid) ligand. The

reporter gene activity was measured and total RLU are shown. The number on top of each bar is the maximum fold induction for that treatment and is the mean of three replicates.

As shown in Figure 15, the CfEcRCDEF/MmRXREF receptor combinations were the best switch pairs both in terms of total RLU and fold induction (compare columns 1-6 to columns 7-12). This confirms Applicants' earlier findings as described in Example 2 (Figures 11-14). The same gene expression cassettes encoding the truncated EcR and RXR polypeptides were also assayed in a human lung carcinoma cell line A549 (ATCC) and similar results were observed (data not shown).

#### EXAMPLE 4

This Example describes a CfEcR-based yeast two-hybrid assay prepared according to the invention. Briefly, gene expression constructs were prepared as follows:

- 4.1 - CfEcR-DEF in pGLIDA: The D, E, and F domains from spruce budworm *Choristoneura fumiferana* EcR ("CfEcRDEF"; SEQ ID NO: 3) were fused to a LexA DNA binding domain ("LexADNABD"; SEQ ID NO: 45) and placed under the control of a GAL1 promoter (SEQ ID NO: 73) in pGLIDA (CLONTECH). This construct results in a LexA:CfEcRDEF fusion that is expressed under the GAL1 promoter.
- 4.2 - CfEcR-CDEF in pGLIDA: The C, D, E, and F domains from spruce budworm *Choristoneura fumiferana* EcR ("CfEcRCDEF"; SEQ ID NO: 1) were fused to a LexA DNA binding domain ("LexADNABD"; SEQ ID NO: 45) and placed under the control of a GAL1 promoter (SEQ ID NO: 73) in pGLIDA (CLONTECH). This construct results in a LexA:CfEcRCDEF fusion that is expressed under the GAL1 promoter.
- 4.3 - MmRXR-A/BCDEF in pB42AD: The A/B, C, D, E, and F domains from mouse (*Mus musculus*) RXR  $\alpha$  ("MmRXRA/BCDEF"; SEQ ID NO: 62) were fused to a B42 transactivation domain ("B42AD"; SEQ ID NO: 53) and placed under the control of a GAL1 promoter (SEQ ID NO: 73) in pB42AD (CLONTECH). This construct results in a B42AD:MmRXR-A/BCDEF fusion that is expressed under the GAL1 promoter.
- 4.4 - MmRXR-DEF in pB42AD: The D, E, and F domains from mouse (*Mus musculus*) RXR  $\alpha$  ("MmRXRDEF"; SEQ ID NO: 22) were fused to a B42 transactivation domain ("B42AD"; SEQ ID NO: 53) and placed under the control of a GAL1 promoter (SEQ ID NO: 73) in pB42AD (CLONTECH). This construct results in a B42AD:MmRXR-DEF fusion that is expressed under the GAL1 promoter.
- 4.5 - CfUSP-DEF in pB42AD: The D, E, and F domains from spruce budworm

*Choristoneura fumiferana* EcR ("CfEcRDEF"; SEQ ID NO: 3) were fused to a B42 transactivation domain ("B42AD"; SEQ ID NO: 53) and placed under the control of a GAL1 promoter (SEQ ID NO: 73) in pB42AD (CLONTECH). This construct results in a B42AD:CfUSP-DEF fusion that is expressed under the GAL1 promoter.

- 5 4.6 - CfEcR-CDEFV in pGLIDA: The C, D, E, and F domains from spruce budworm *Choristoneura fumiferana* EcR ("CfEcRCDEF"; SEQ ID NO: 1) were fused to a LexA DNA binding domain ("LexADNABD"; SEQ ID NO: 45) and to a VP16 transactivation domain ("VP16AD"; SEQ ID NO: 49) at N-and C-termini respectively, and placed under the control of a GAL1 promoter (SEQ ID NO: 73) in pGLIDA (CLONTECH). This construct results in
- 10 a LexA:CfEcR-CDEF/VP16 fusion that is expressed under the GAL1 promoter.

4.7 - pSH118: A reporter plasmid comprising a  $\beta$ -galactosidase gene (LacZ; SEQ ID NO: 74) under the control of an 8X LexA operon response element ("8XlexAop"; SEQ ID NO: 75) operably linked to a minimal TATAA region from the GAL1 promoter (p8op-lacZ from CLONTECH) was used to monitor inducible gene expression.

15

**Yeast Cells:** *Saccharomyces cerevisiae* yeast cells (EGY48; CLONTECH) were used in these experiments. Cells were maintained in liquid culture media with the appropriate drop out amino acids. Standard methods for culture and maintenance of the cells were followed.

- Transfections:** DNAs corresponding to the various switch constructs outlined in Examples
- 20 4.1 through 4.7 were transfected into the yeast cells following the protocol described by Ito et al., 1983 (J. Bacteriol., 153:163-168).

**Ligands:** 20-hydroxyecdysone (20E), Ponasterone A (PonA) and Muristerone A (MurA) were purchased from Invitrogen and Sigma Chemical Company. The four non-steroids N-(2-ethyl-3-methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-t-butylhydrazine (GS<sup>TM</sup>-E), N-tert-

25 butyl-N'-(3-methoxy-2-methylbenzoyl)-N-(3,5-dimethylbenzoyl)hydrazine (RH-2485), N-tert-butyl-N'-(4-ethylbenzoyl)-N-(3,5-dimethylbenzoyl)hydrazine (RH-5992), and N-tert-butyl-N,N'-dibenzoylhydrazine (RH-5849) are synthetic stable ecdysteroids synthesized at Rohm and Haas Company. All ligands were dissolved in DMSO and the final concentration of DMSO was maintained at 0.1% in both controls and treatments.

### 30 Results

DNA constructs pSH118, CfEcRDEF, and MmRXRA/BCDEF, MmRXRDEF, or CfUSPDEF were transfected into yeast cells. In addition, DNA constructs pSH118, CfEcRCDEF, and MmRXRA/BCDEF, MmRXRDEF, or CfUSPDEF were transfected into yeast cells. As a control, DNA constructs pSH118 and CfEcRCDEV were also transfected

into yeast cells. A colony from each group of transformants was grown in the liquid culture in the presence of 0, 0.04, 0.2, 1, 5 and 25  $\mu\text{M}$  GS<sup>TM</sup>-E. The reporter activity was measured as described above 4 hours after adding ligand. Total RLUs were calculated and the numbers on the bars of Figure 16 show maximum fold induction observed for that group (see 5 Figures 16 and 17).

As shown in Figures 16 and 17, transactivation by CfEcR:CfUSP and CfEcRCDEFV combinations are ligand independent, whereas CfEcR/MmRXR combinations are ligand dependent. CfEcRDEF/MmRXRDEF performed the best of any combination tested. This yeast assay closely reflects the performance of these switch formats in mammalian cells. The 10 background levels are lower and induced levels are higher, and as a result, the fold induction is high, approximately 1800-fold. This number is much higher than the fold inductions observed by Tran et al., 2001 (Mol. Endo., 15:1140-1153; and International PCT patent application publication WO 01/61350A1). Thus, the dose-response of GS<sup>TM</sup>-E on the gene regulation system described herein in yeast cells appears to be similar to that observed in 15 mammalian cells.

The best performing transformed yeast strain from the analysis above comprising CfEcRDEF:MmRXRDEF was used to determine the induction activity of three steroid ligands (20E, PonA, MurA) and four non-steroid ligands (GS<sup>TM</sup>-E, RH2485, RH5992, RH5849) in this yeast-based format. The yeast cells were cultured in the presence of 0, 0.1, 20 1, 10, 50 and 100  $\mu\text{M}$  ligands for four hours and the reporter activity was measured as described above. Total RLUs were calculated and the numbers on the top of the bars of Figure 18 show the maximum fold induction for that group (see Figures 18 and 19).

As shown in Figures 18 and 19, in the transformed yeast strain comprising CfEcRDEF:MmRXRDEF, GS<sup>TM</sup>-E was able to induce gene expression better than any other 25 ligand tested, and was followed in performance by RH-2485 and RH5992. The three steroid ligands and the remaining non-steroid ligand RH5849 did not show a significant response. The fold induction activity is similar to that observed by Tran et al., 2001 (Mol. Endo., 15:1140-1153; and International PCT patent application publication WO 01/61350A1) as well as in mammalian cells.

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The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the

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scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

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